Yield and phytotoxicity responses of subterranean clover (*Trifolium subterraneum* L.) sprayed with different herbicides for broadleaf weed control

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A key factor for integrating subterranean clover into pastoral systems is its success in the establishment year. With appropriate management, establishment ensures productivity and persistence in future years. This thesis investigated the relative herbicide tolerance of subterranean clover at the seedling stage for pasture establishment in New Zealand rain-fed environments. This involved quantification of the field response to herbicides, at different seedling development stages of subterranean clover cultivars. Four experiments, two arranged as split-plot, and two as split-strip-plot, were established in Canterbury, New Zealand in autumn 2016. Emergence of Experiments 1 and 2 occurred in late-March, while Experiments 3 and 4 were delayed by lack of rainfall. A cultivar*herbicide interaction was identified in all experiments, confirming that cultivars were different in their response to herbicides.

The cultivar 'Narrikup' showed the greatest herbicide tolerance to imazethapyr across all experiments, with sown clover yields of 2600-3500 kg DM/ha. All *yanninicum* cultivars ('Monti', 'Napier', and 'Trikkala') were not suited to the environmental conditions of the season and failed to persist following herbicide applications. The subterranean clover subspecies *brachycalycinum* cultivar 'Antas' showed variable herbicide tolerance, with responses of developmental delay as well as yield depression. The white clover control was consistently the lowest yielding at <1000 kg DM/ha, with no response to herbicides.

Subterranean clover cultivars had visible phytotoxicity responses to imazethapyr which were related to plant pubescence. The phytotoxicity scores were correlated to yields within each experiment. 'Whatawhata', 'Woogenellup', and 'Narrikup' cultivars showed the greatest tolerance and benefit from imazethapyr, with total clover yields of 3500-4500 kg DM/ha for the growing season, >2000 kg/ha more than their unsprayed unweeded controls.

For cocksfoot/clover mixtures only 'Narrikup' had no reduction in total dry matter yields compared to unsprayed unweeded controls when imazethapyr was applied. Cocksfoot productivity was slowed by imazethapyr up to 24 weeks after application, with yields 750 kg/DM lower than the unsprayed unweeded controls. Pastures recovered to be no different in November, and cocksfoot can be expected to continue to provide summer grazing after the annual clovers set seed. The early reduction in cocksfoot productivity allowed >20% increases in the clover component of imazethapyr treated swards.

Experiments 3 and 4 found that the ALS-inhibiting herbicides imazethapyr and flumetsulam, and photosynthesis-inhibitor bentazone were the least damaging herbicides to subterranean clover. Experiment 3, where plants were treated at the 1-2 trifoliate leaf stage had higher subterranean clover yields when compared to Experiment 4, where herbicide was applied at the 4-6 trifoliate leaf stage. The early seedling herbicide application had less impact on yields than prolonged competition from weeds. Combined sown+resident clover yields showed that sowing cultivar mixes can improve subterranean clover herbicide tolerance and increase total yields. Both application times for Imazethapyr, flumetsulam, and bentazone had mean total clover yields the same as or greater than the unsprayed unweeded controls. 2,4-DB had a negative impact on the development of all subterranean clover plants. For the remaining herbicides bentazone + MCPB, bromoxynil + diflufenican, and MCPB, 'Antas' and resident 'Woogenellup' were susceptible to developmental delays as a result of application, while 'Denmark' and 'Narrikup' were less adversely affected.

Imazethapyr + saflufenacil, and glyphosate treatments killed all vegetation and left ground bare in all experimental applications, confirming they are unsuitable for use in subterranean clover-containing pastures. This research confirmed a cultivar*herbicide interaction to a range of herbicides, and identified imazethapyr, flumetsulam and bentazone as suitable for use during establishment of subterranean clover based pastures. Longer term effects, such as those on subsequent regeneration and further investigations into effects on development, as well as the apparent *brachycalycinum* susceptibility are advised.

Keywords: *Trifolium subterraneum* L., subterranean clover, New Zealand, dryland pastures, broadleaf weeds, herbicides, cocksfoot, phytotoxicity, 2,4-DB, bentazone, bromoxynil, diflufenican, flumetsulam, imazethapyr, MCPB, saflufenacil

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1 GENERAL INTRODUCTION

Dry hill and unirrigated lowland pastures that receive less than 800 mm of annual rainfall make up 10.7% of total land area in New Zealand, which is around 3-4 million hectares (Brown & Green 2003; Monks *et al.* 2016). There are additional areas which have dry sunny (north and west) slopes in summer moist environments that are often exposed to long periods of drought as a result of high levels of potential evapotranspiration. These dry areas are not conducive to the persistence and productivity of the common perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.), which can be severely affected when rainfall is below 600-700 mm per annum (Knowles *et al.* 2003; White *et al.* 1999). Low pasture production, especially in the cool early spring period can affect live-weight gains, particularly during lactation, and therefore limit farm production (Ates *et al.* 2006; Mills *et al.* 2008). In New Zealand dryland pastoral systems, the selection of appropriate pasture species requires consideration of their rainfall requirements and persistence under drought conditions. In these summer dry areas, annual legumes are a potential alternative to provide persistent pastures capable of producing consistently high quality feed (Monks *et al.* 2016).

Sown clover species are particularly vulnerable to competition at establishment, when resources are limiting and competition for light is high (Ates *et al.* 2006; Frame & Newbould 1986; Smetham 2003). With successful early weed control, competition is reduced, and successful establishment of the sown pasture species is ensured. Where possible, conventional cultivation methods offer the opportunity to bury weed seeds through ploughing with a light harrow pre-sowing to deal with potential emerging weeds (Evers *et al.* 1993). However if weeds have been poorly controlled during seedbed preparation, they often manage to re-establish before the sown seed can germinate. This causes heavy competition and in some cases can lead to heavy losses, impeding optimal pasture establishment (White *et al.* 1999). Post-emergence, additional weed seedlings will emerge and compete with the establishing pasture. In these situations, grazing management is often used to control weed growth, but on occasion there may also be a need for chemical control (Evers *et al.* 1993). Growers prefer selective post-emergent herbicide options for weed control over pre-emergent options to avoiding outlaying expenses where they may not be required (Green *et al.* 2006) and few exist for new pastures anyway.

1.1 Annual clovers

Annual clovers are sown, or re-establish from seed each autumn. They reach their peak growth during spring, before setting seed and senescing in the late spring/early summer period depending on species and cultivar. By doing this, they have the ability to avoid summer dry periods, with the aim of successful re-establishment the following autumn to continue their life-cycle (White *et al.* 1999).

Annual legumes are widely used in neutral-alkaline soils across the low-medium rainfall areas of southern Australia and the southern subtropics (Nichols *et al.* 2007). The biggest advantage these annual clovers provide is their potential to increase pasture quality and quantity over the cooler months when the growth of perennial clovers is slower. The inclusion of annual clovers within a mixed sward also serves to increase diversity within the pasture, and can widen its adaptability to a range of environmental conditions. They can provide rapid early spring growth, raise soil and pasture quality through nitrogen fixation, improve drought tolerance, and extend spring grazing periods before seed set to regenerate the seed bank to provide seed for several years (Nori 2013).

1.2 Subterranean clover

Subterranean clover, *Trifolium subterraneum* L. (hereafter subterranean clover) is the most commonly sown annual legume species in New Zealand (Monks *et al.* 2016; Stewart *et al.* 2006). It is considered the most important, and appropriate self-regenerating annual clover species commercially available within New Zealand and is adapted to summer dry environments (Lucas *et al.* 2015; Monks *et al.* 2016; Smetham 2003). Two key factors which have led to the widespread adoption of subterranean clover are its ability to persist despite heavy grazing pressure; and the existence of a suite of cultivars with different photothermal time requirements for flowering, which allows its introduction in a range of highly variable environments (Nichols *et al.* 2007). Under appropriate management, it can provide significant gains in both quality and dry matter yields of pastures where the performance of perennial legumes is limited by harsh summer conditions (Dodd *et al.* 1995; Smetham 2003). With inclusion of nitrogen fixing subterranean clover in a pasture, productivity of other components can increase, subsequently enhancing animal gains and farm profits (Sheath & Macfarlane 1990). It has substantially faster growth than lucerne and perennial grasses through late winter and early spring, and under proper management is capable of high quality forage production through this period of high animal demand (Moot *et al.* 2003; Smetham *et al.* 1994; Widdup & Pennell 2000). As an annual species, successful growth in the establishment year is essential to provide a seed set which ensures the persistence and success of the pasture in years to come.

The majority of subterranean clover research applied in New Zealand is based on Australian farming systems, their productivity, management, and environment. Australian systems are generally a Mediterranean type climate, used in pasture, cropping rotations or pure swards (Puckridge & French 1983; Smetham 2003). New Zealand has a more temperate climate, so the application of Australian management techniques and transfer of technical information can be compromised. To date, the range of subterranean clover cultivars commercially available in New Zealand has been reliant on Australian seed harvests, and their ability to enter through the biosecurity requirements, without selection through New Zealand based field research (Lucas *et al.* 2015).

New Zealand subterranean clover research has so far focused predominantly on hill country systems, which need legume species able to survive summer dry periods, cultivar choices and associated fertiliser inputs for optimal establishment (Dodd *et al.* 1995; Hoglund 1990; Sheath & Macfarlane 1990; Smetham 2003). Currently there is little research into more recently developed herbicides, and none specifically for use on subterranean clover in New Zealand. Monks *et al.* (2016) stated a key area of consideration for increased uptake of subterranean clover in New Zealand is the investigation of agronomic solutions surrounding its introduction, and reseeding in pre-existing pastures. This research can be seen as a start to this process. Comprehensive management of subterranean clover in New Zealand requires agronomic studies into all aspects of establishment.

The aim of this thesis is to provide herbicide recommendations suitable for use with different subterranean clover cultivars in New Zealand, and collate them in context for integration into New Zealand dryland pasture establishment recommendations.

This thesis is structured in seven chapters (Figure 1.1). Chapter 2 reviews the literature, outlining the life cycle of subterranean clover, presents current knowledge of herbicides for establishing clovers in pastures, explains methods of quantifying herbicide tolerance and describes the mechanisms of action for the herbicides used in the experiments.

Chapter 3 deals with Objective 1: to evaluate visible and physical differences in cultivar tolerance to two herbicides and identify potential explanations.

In Chapter 4, Objective 2 is: to investigate the relative tolerance of a subterranean clover/cocksfoot pasture mix to the same two herbicide treatments.

Chapters 5 and 6 investigate Objective 3: to quantify any differences in tolerance of four subterranean clover cultivars to eight herbicide treatments applied at two different seedling growth stages.

Chapter 7 collates the results from all experiments in context for integration into New Zealand dryland pasture establishment recommendations. The experimental results provided are unique to the experiments conducted and come with the *caveat* that only registered herbicides should be used on subterranean clover based pastures in New Zealand.

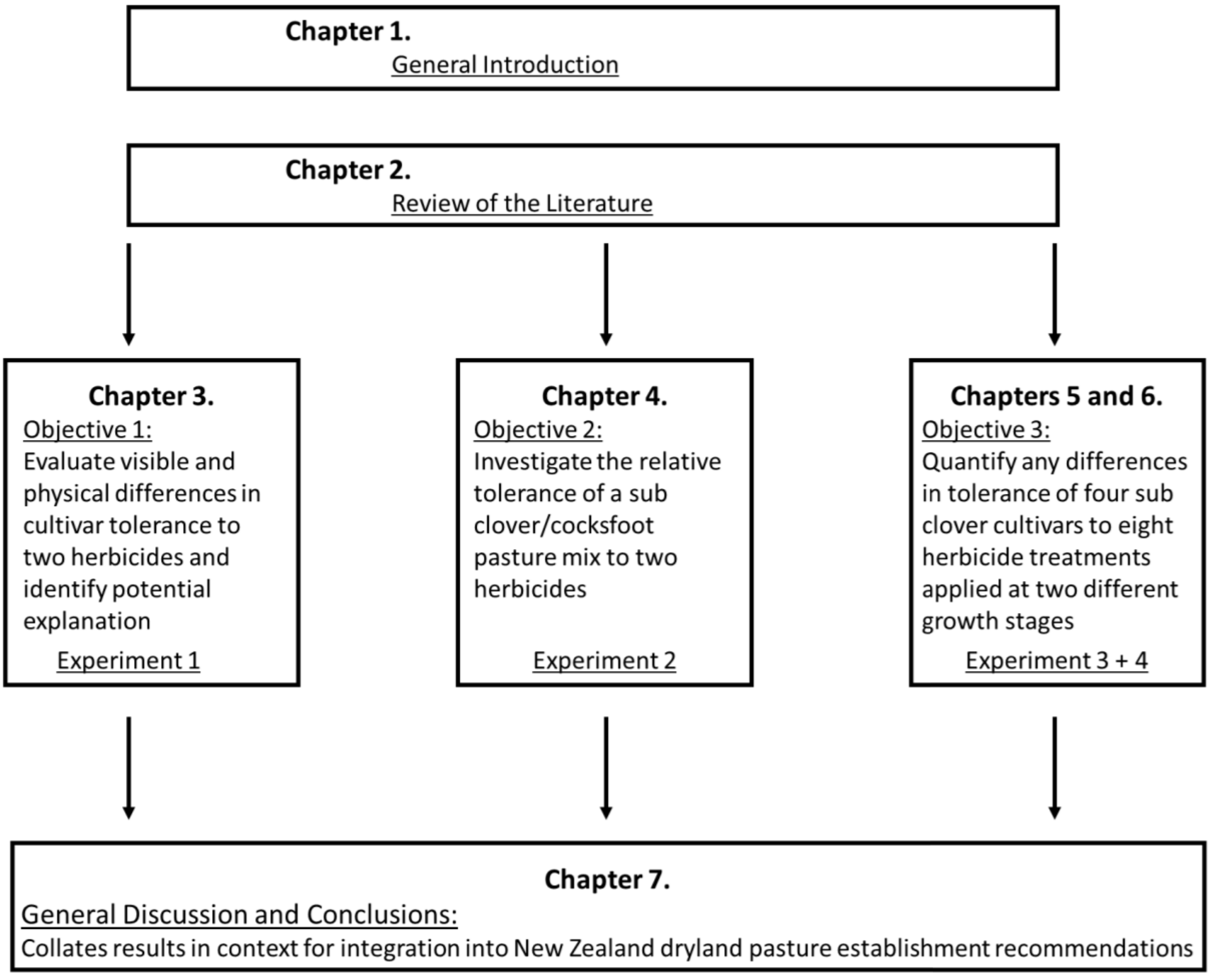

Figure 1.1 Flow diagram of thesis structure

2 REVIEW OF THE LITERATURE

This chapter reviews current literature to explain subterranean clover, its life cycle, behaviour in the New Zealand environment, and how it is impacted by broadleaf weeds. Also discussed are the herbicides recommended in New Zealand for broadleaf weed control in white clover pastures, their modes of action, and currently available information on their phytotoxicity effects in subterranean clover.

2.1 Subterranean clover

The subterranean clover species has primarily been divided into three main subspecies on the basis of edaphic adaptation (Smetham 2003). Of the three subspecies, *T. subterraneum* L. ssp. *subterraneum* is the most common, and versatile, found in acid to neutral, well-drained soils with annual rainfall ranging <100-1540 mm. *T. subterraneum* L. ssp. *brachycalycinum* is best in higher pH (>5.8), stony or ruderal soils, but can also tolerate heavier clay soils. *T. subterraneum* L. ssp. *yanninicum* prefers acid to neutral, poor draining soils, and is successful in areas with winter waterlogging.

2.1.1 Life cycle

Germination of sown subterranean clover seed, or seed from within the seed bank, occurs when rain begins in autumn. Vegetative growth occurs throughout winter into early spring, when the switch to reproductive development initiates, but timing of this differs depending on cultivar (Smetham 2003) (Table A.1). Flowering occurs early in spring, enabling the development and maturation of the seed containing burrs before moisture stress impedes growth (Smetham *et al.* 1994). Throughout the dry summer period, the subterranean clover persists as seed below the surface in buried burrs, germinating with the occurrence of rains in autumn, to complete itis life cycle (Dodd *et al.* 1995). The soil seed bank often maintains large reserves of seed, utilised when annual seed production is lower than needed for optimal regeneration (Frame & Newbould 1986). Such autumn sown pastures often experience exacerbated weed problems in milder seasons, where clover establishment is often poor and/or slow. This can require chemical control post seedling emergence to reduce competition for resources (Frame & Newbould 1986).

2.1.2 Effect of season on subterranean clover success

The time of the autumn rain to start the season and allow subterranean clover germination to occur determines the seasonal yield potential of the clover. With an early break to the season, subterranean clover is more competitive than balansa and white clover, and can often almost entirely suppress weeds. When the break is later, the weeds which are better adapted to the environment have an advantage over the germinating clover, and can compromise establishment success. When rain occurs in early autumn, seeds germinate early, allowing an optimal season for clover growth, with mid-September yields of up to 7000 kg DM/ha reported for clover that germinated on 7 March in Canterbury, New Zealand (Moot *et al.* 2003). However successive later breaks decreased yield potential, down to 1800 kg DM/ha by mid-September for a break on 7 May. This difference in yield potential is a function of temperature limiting the thermal time requirement for development, and the phyllochron (leaf appearance rate), with later germinating plants emerging into lower temperatures, which causes a reduction in growth rates (Richardon 2003). The thermal time requirements (°Cd) with T_b (base temperature) set at 0 °C for the appearance of the first trifoliate leaf for subterranean clover from radicle protrusion is between 216 and 251 °Cd depending on the cultivar (Moot *et al.* 2003), with an average of 229 °Cd. The average phyllochron for subterranean clover has been found to range from 63 to 73°Cd, with a mean of 67 °Cd (Richardon 2003). The thermal time requirement of subterranean clover to reach the six trifoliate leaf stage was calculated to be 434 °Cd. With later breaks to the season, or delayed sowing times, plants emerge into cooler average temperatures, causes exponential increases in the cumulative number of days required to reach specific developmental stages. This results in an increase of chronological time required for seedlings to reach a critical size. A further disadvantage of late opening rain is the increase in time before subterranean clover can be safely grazed. When rain occurred on February 1, only 23-26 days were calculated to be required for growth before safe grazing could occur across New Zealand. However, when rain occurred on May 1, causing late germination and slow plant growth, the time required for safe grazing was estimated as between 39 days in Napier, to 102 in Alexandra (Moot *et al.* 2003).

2.1.3 New Zealand management

The 'MaxClover' grazing experiment which ran at Lincoln University, Canterbury, New Zealand from February 2002 until 2011 identified cocksfoot (*Dactylis glomerata*) subterranean clover mixtures as the most productive in a dryland environment. Yields of the cocksfoot subterranean clover pastures were 8700-13000 kg DM/ha annually, with a subterranean clover component of 2400-3700 kg DM/ha in six of the nine experimental years (Mills *et al.* 2014). Further to this, a 2014 field trial at Lincoln University investigated cocksfoot x 10 different subterranean clover cultivars, and found that cultivars 'Antas' and 'Narrikup' produced the highest total early spring DM yields in September, with 758±62 kg DM/ha (Lucas *et al.* 2015). In November, 'Antas' was again one of the highest yielding cultivars, along with 'Woogenellup' and 'Leura', producing yields of 2712±243 kg/ha. 'Narrikup' was one of the lowest yielding cultivars in this later harvest, along with 'Denmark', 'Rosabrook' and 'Monti', with 1465±244 kg/ha. Such trials are affected by broadleaf weed ingress both at establishment, throughout the season, and during regeneration, and a lack of knowledge of which herbicides can be used specifically on subterranean clover in New Zealand means weed problems are not easily rectified (Lucas *et al.*

2015; Mills *et al.* 2014). Recommendations for white clover seedlings are not always appropriate due to the different environmental conditions subterranean clover is routinely sown in.

2.2 Broadleaf weeds

Weeds in pastures and crops are capable of outcompeting the sown species, lowering yields, increasing labour and cost requirements, and decreasing overall outputs (Kelton & Price 2011). Broadleaf weeds are dicotyledonous plants (two leaves), generally identified by the presence of veins in the leaves, and flower parts present in fours, fives, or multiples of (Ross & Lembi 2008). Many agricultural weeds are annual plants, which thrive in frequently disturbed areas, have quick vegetative growth from seed, and can reseed year after year, and build up large seed stores within the soil (Kelton & Price 2011). Broadleaf weeds have been found to affect crop growth from the seedling stage by decreasing the proportion of red light which reaches the sown seedlings. With increases in weed leaf area, red light levels decrease exponentially, and the decreased ratio of red to far-red light impacts the seedling growth of other plants. With successful herbicide application, red light exposure of sown seedlings will increase as weeds are removed, meaning sown species are subject to less competition for light for photosynthesis (Cressman *et al.* 2011). As swards become denser, the ratio of red/far-red light beneath the canopy increases, so even if weeds are removed, the subsequent establishment of a dense canopy as a result of less species competition can have a similar effect below the canopy. Plants that receive less red light become carbohydrate deficient, and increase their spread to reach new areas of light, rather than growing upright towards the light (Cressman *et al.* 2011; Taiz & Zeiger 2010).

2.3 Herbicides

The ideal, desired outcome following herbicide use is that only the weed is killed. Modern herbicides are extremely successful due to the development of selective phytotoxicity actions. Selectivity is relative however, and can be dependent on dose and application rate, which is why guidelines are provided on herbicide labels for target and safe species (OEPP/EPPO 2014). Successful weed control requires uptake of the herbicide by the non-desirable plant, its movement within the plant to the target site, and metabolism of the active ingredient, which triggers a cascade of secondary metabolic events, leading to the cessation of growth and senescence of the target plant (Cobb & Reade 2010). Selectivity can be based on morphological differences between plant species, so the more similarities between a crop and its resident weed species, the more likely it is that the herbicide will have higher uptake than intended in the non-target plants (Kelton & Price 2011). Often, with increased herbicide efficacy against broadleaf weeds comes an increase in the potential for unintended collateral effects, generally in the form of damage to the sown species (Frame & Newbould 1986; Streibig 2003). Altering the formulation of a herbicide can exploit plant differences in absorption and uptake, as well as the relative rates of both long and short distance transport systems within plants.

Plants are extremely capable of detoxifying unknown chemicals (xenobiotics) which they encounter in their environment (Cobb & Reade 2010). Differences in the metabolic capacity of the detoxifying enzymes of plants, and varying *in planta* target sites are the key factors in modern herbicide selectivity (Streibig 2003). Damage as a result of herbicide application can also occur despite all precautions if plants are suffering abiotic or biotic stress, as it augments the effect these stresses already impose on the plant (Buchanan *et al.* 2015; Frame & Newbould 1986). This has been observed before in subterranean clover where annual rainfall at the experimental site in Wagga Wagga, Australia in 1989 was 704 mm, 35% above average with favourable conditions, and 564 mm in 1990 with drier late spring weather, but available soil moisture over the winter-early spring period was similar for both years. When treated with the grass killer simazine (a.i. 0.63 and 1.25 kg/ha), a residual herbicide which controls both grasses and dicotyledons during germination, 'Karridale' showed a yield loss in spring of 74-92% in 1989, compared with 38-42% in 1990, while 'Trikkala' yields were reduced by 38% in 1989 and 16% in 1990. (Dear *et al.* 1992). These large variations in cultivar yields for identical treatments and application times in the same location across years with different weather conditions shows the impact fluctuations in environmental conditions can have on herbicide tolerance (Evers *et al.* 1993).

An increase in susceptibility of already stressed crops is largely due to how the rates of herbicide absorption and metabolism respond to stress. Since herbicide absorption is a physical process (diffusion), environmental factors that stress the plant are unlikely to cause large reductions in herbicide movement from the leaf surface into the plant cell. The capacity of the plant to successfully metabolize the herbicide once it has entered however, preventing its passage to the target site, can be greatly affected by environmental changes that induce stress (Roberts 2000).

The physical and chemical composition of the plant cuticle, the environment, and the chemical formulation of the herbicide itself are all factors which influence herbicide efficacy (Streibig 2003). Some herbicides are initially inactive, requiring conversion to their metabolically active form within the plant before phytotoxicity will occur. Ultimately, the effectiveness of any herbicide is dependent on the quantity of active ingredient able to reach the target site. There are many barriers between the outside of the leaf cuticle and the site of action, through which the herbicide must pass (Kelton & Price 2011). Chemicals can have poor permeability through plant cuticles and membranes when applied alone, especially if cuticles are waxy or plants have high trichome density. To negate this barrier, herbicides are often mixed with an adjuvant to guarantee successful uptake. An adjuvant is a biologically inactive compound which aids the entry of the chemical into the lipophilic profile of the cuticle. Once in the plant, most adjuvants are assumed to have no effect on transport and metabolism of the active ingredient unless specifically stated (Cobb & Reade 2010; Streibig 2003).

2.4 Herbicide phytotoxicity

A compound is said to be phytotoxic if it has an injurious effect, be it temporary or long-lasting, on plant growth. Phytotoxicity assessments are used when determining plant-herbicide compatibility to report on the efficacy of assessed herbicides on the sown species. The basic principles used when assessing the phytotoxicity of a given compound are the same, irrespective of the type of compound. Effects of herbicide phytotoxicity can present at emergence (if pre-emergence products are applied), throughout crop growth and developmental stages, or are expressed at the harvest stage. These effects can often be temporary and rectify themselves without impact on overall crop productivity. Alternately they can last longer and impede the productivity of the crop. Symptoms can occur on individual parts of the plant (roots, shoots, leaves, flowers, seeds), or affect the entire plant and should always be accurately recorded, preferably with photographs as the development of symptoms may not always be immediately apparent (OEPP/EPPO 2014).

Phytotoxicity assessments can be absolute: numbers of plants at a certain development stage/showing a particular visual toxicity symptom, heights, lengths, diameters, weights of sample plants or organs; or scored on a visual scale of 0-100%: estimates of deformities, discolouration, comparisons of crop productivity with the control (Kroschel 2001). Damage within the sward of a mixed pasture can often go unnoticed as it is a dynamic system, with many factors affecting changes that cause difficulties in elucidating the true cause of a symptom (Frame & Newbould 1986).

The European Weed Research Society has a scale for visual ratings of herbicide phytotoxicity (Table 2.1). This can be used to estimate the effect of herbicide treatments on plots of sown species relative to unsprayed control plots. This scale is an internationally recognised score of visual phytotoxicity symptoms presenting as a result of herbicide treatment. A herbicide treatment score greater than 5 is not expected to have any commercial viability as a herbicide, as the damage caused to non-target plants is considered greater than any weed control achieved (more than 50% of the crop visibly damaged/reduced). Morphological deviations of plants from the observed controls (symptoms such as leaf curling, rolling, stunting or elongation of plant organs, changes in plant size or volume, plant colour changes, chlorosis, whitening, changes in colour intensity, browning, reddening, and necrosis of whole plants, or parts) are accounted for through changes in the EWRS scores of plots (OEPP/EPPO 2014).

Table 2.1 European Weed Research Society (EWRS) phytotoxicity damage score

Score	Damage symptoms
1	No damage / healthy plants
2	Very mild symptoms
3	Slight, but clearly visible symptoms
4	Severe visible damage, e.g. chlorosis, which do not lead to a negative effect on yield
5	Thinning, severe chlorosis, leaf burn or suppression, some yield reduction expected
6	Above commercial threshold, symptoms range from severe damage to total plant death
7	
8	
9	

(Kroschel 2001; Streibig 2003)

2.5 Post-emergence herbicides

The commonly used post emergence broadleaf herbicides in pasture scenarios are generally phytotoxic to legumes. This is a result of their closer relationship to dicotyledonous weeds than most cropping species. As a consequence, herbicide application often leads to a reduction in legume herbage yields and this loss of productivity reduces the potential total fixation of nitrogen (Sandral & Dear 2005). There has been some research in subterranean clover on establishment and weed ingress effects, however none of this was done in a New Zealand environment and the research of late is looking towards genetic modification options as a solution (Dear *et al.* 2003). This is not viable in the current New Zealand environment because of strict anti-GMO laws. The bulk of work on subterranean clover tolerance to herbicides has been carried out in Australian environments, on pure subterranean clover swards since the early 1990s. Sandral and Dear (2005) concluded that an increased understanding of herbicide tolerance is required to aid in species selection for pastures, and careful selection of herbicides is paramount when spraying a legume based pasture to prevent losses. They advised that further work be carried out to determine differential cultivar tolerances, and push for the selection of cultivars for improved herbicide tolerance. Therefore it is important to understand the effects of broadleaf herbicides commercially available within New Zealand on subterranean clover-containing pastures. Recommendations are often given on herbicide labels as safe for ‘clover’, but this usually refers to white clover, and occasionally red clover, with no mention of annual clovers. Differences in herbicide phytotoxicity to clover species indicate that post-emergence herbicides need a separate evaluation for each species and potentially for different cultivars within that species before one is recommended for viable use (Evers *et al.* 1993).

Research on white clover has found that seedlings rarely emerge uniformly, which leads to a varying range of developmental stages upon herbicide application rather than at a uniform trifoliate leaf stage as often recommended for tolerance on product labels. As a result, there can often be an impact on clover development rate post-herbicide application, and plant death can occur (Brock & Hay 2001). Investigations into application timing for the non-selective herbicide paraquat (a.i. 1.5 L/ha) on subterranean clover containing pastures (no cultivars specified) found that late winter (July) applications provided a higher proportion of legume content within the sward than applications earlier in winter with 81% subterranean clover, compared to 59%. However these proportional increases were correlated with 500 kg/ha decreases in spring subterranean clover biomass from 5000 kg/ha (Scammell & Ronnfeldt 1998).

Historically, the selective herbicides MCPB and 2,4-DB have been widely recommended and used for broadleaf weed control within establishing and established legume-based pasture swards (Thimann 1939). However despite being safe on clover seedlings, these herbicides have limited efficacy against broadleaf weeds, and are often boosted with herbicides less selective to clover, such as MCPA and 2,4-D, or have been superseded by newer herbicide formulations (Cobb & Reade 2010; Frame & Newbould 1986). Evans *et al.* (1989) demonstrated that the phenoxy herbicides 2,4-DB and MCPA can cause losses in yield of up to 62%, with differences among subterranean clover cultivars. Bromoxynil (a.i. 0.3 kg/ha) was the most tolerated of the evaluated herbicides, with dry matter production reductions of 5.4-8.9% in 'Seaton Park' and 'Karridale', 15.7% in 'Woogenellup', 27.7% in 'Trikkala', and 38.9% in 'Mt Barker'. Subsequent research by Dear *et al.* (1995) found that mixed subterranean clover swards are bromoxynil tolerant (a.i. 0.3 kg/ha), suffering yield losses of up to 34%, with the fastest recovery after treatment, at a rate of 80 – 120 kg DM ha/day, with 'Trikkala' the most tolerant cultivar. In south-eastern Australia, annual legume-based pastures which have a high component of subterranean clover are widely treated for weed control using herbicides which contain bromoxynil (3,5-dibromo 4-hydroxy benzonitrile). This can be on its own, or in herbicide mixtures with chemicals such as MCPA or diflufencian for broadleaf weed control (Dear *et al.* 2003). However, use of straight bromoxynil (standard packaging, a.i. 400 g/L) is not recommended in New Zealand on new or established pasture due to the difference in climate; application is not recommended for use on crops undersown with legumes or clover. However, bromoxynil + diflufenican mixtures (a.i. bromoxynil 250 g/L, diflufenican 50 g/L) are commercially available (Table A.2) and recommended for use on establishing pastures which contain clover (Novachem 2016).

With this information, it can be seen that there is a need for specific investigation into viable methods of broadleaf weed control in subterranean clover-based pastures in New Zealand environments. Chemical structures for all discussed herbicide active ingredients are available in the Appendix (Table A.2).

2.5.1 Auxin-type herbicide action

The phenoxyacetic acids MCPA (2-methyl-4-chlorophenoxyacetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) are synthetic indole-3-acetic acid (auxin, or IAA) compounds, and were originally developed as potential agents for chemical warfare, and only later realised to have agricultural potential. The phenoxy herbicides were rapidly accepted for use worldwide since 2,4-D was registered for commercial use in 1945 (Kirby 1980). They are still among the most commonly used herbicides today (Cobb & Reade 2010). Auxin is a plant growth hormone involved in cell division, differentiation, and elongation. It acts on plant physiology at both the organ and whole plant level (Buchanan *et al.* 2015; Fedtke & Duke 2004). Auxin-type herbicides are successful because of their high level of selectivity. However, this is species-dependent, as different hormones and chemical compounds can have vastly different effects and actions across species. As a rule, increases in concentration will lead to increases in phytotoxicity. However in the case of auxin-types, this can require a large increase in concentrations, and what causes huge disruption to one plant may have no noticeable effect on another (Cobb & Reade 2010; Pillmoor & Gaunt 1981).

The herbicide activity of artificial auxins occurs in three distinct phases:

1. Stimulation – despite the role of auxin as a growth stimulant, constant high *in planta* concentrations will cause an imbalance in the plant hormone levels. Root growth is inhibited by high auxin levels, while these same high concentrations stimulate leaf growth. The plant essentially 'grows itself to death' above ground by having inadequate roots below ground which are unable to sustain the increased above ground growth. These high hormone concentrations increase the synthesis of specific mRNA and proteins, prompting rapid shoot growth to occur. The hormone action of auxin occurs unidirectionally from source (leaves=synthesizing tissues) to sinks (meristems and roots). Auxin herbicides (artificial auxins) follow the same metabolic pathways as natural IAA. However because they are not identical, they are poor substitute substrates for the uptake enzymes involved in the downstream production of gibberellins and cytokinins. These lead to the production of ethylene and abscisic acid promoting senescence which leads to heavily reduced metabolic rates and high internal concentrations of hormones (Grossmann *et al.* 2001).

2. Inhibition – as high hormone concentrations continue, high ethylene concentrations stimulate abscisic acid production via auxin-induced ACC synthase. Abscisic acid is a growth inhibiting hormone, and high concentrations close the stomata and halt the internal metabolic processes of the plant. Inhibition of plant growth as a consequence of supra-optimal concentrations of auxin is predominantly a result of increased ethylene production induced by auxin. Once a

critical auxin concentration accumulates within a plant tissue, ethylene and abscisic acid production occurs, inhibiting cell division and growth (Cobb & Reade 2010; Taiz & Zeiger 2010).

3. Decay – once growth has ceased, the affected plant is unable to continue photosynthesizing, however cells in the meristem and cambium continue to grow and divide, producing new tissue. This tissue is not produced in synchrony with the requirements of the plant, creating distended plants, with curling and swelling areas. Chloroplasts will swell and reduce intracellular compartmentation within leaves. The phytotoxic effect of auxin herbicides is a result of prolonged high concentrations in plant tissue, causing eventual deregulation of metabolic processes and growth, leading to decay (Cobb & Reade 2010; Grossmann *et al.* 2001; Pillmoor & Gaunt 1981). Abscisic acid and ethylene will eventually cause plant tissue to senesce and decay (Fedtke & Duke 2004).

Visibly, shortly after application of an auxin-herbicide, leaves of affected plants will become dark green and wither, while stems twist and curl. Petioles can develop epinasty (turn downwards). Plants will eventually become necrotic and decay, indicating that all available energy reserves have been exhausted (Fedtke & Duke 2004; Kirby 1980). The occurrence of events leading to final senescence is dependent on the age and physiology of exposed tissues, and can differ among species (Cobb & Reade 2010; Pillmoor & Gaunt 1981).

Auxin growth regulator herbicides are used for control of annual, simple perennial, and creeping perennial broadleaves in sown crops as well as in non-crop situations. All are organic acids which take on a negative charge after ionization of acids and salts. Esters are hydrolysed to acids or salts in both plants and soils. Injury to off-target vegetation is a major problem associated with these herbicides (Fedtke & Duke 2004).

2.5.1.1 2,4-DB

2,4-DB (4-(2,4-Dichlorophenoxy)butanoic acid) is an auxin-type herbicide, out of patent, developed from 2,4-D (PubChem 2016a). Dow AgroSciences 2,4-DB will be used in this study which contains 400 g/L 2,4-DB as the sodium salt in a soluble concentrate (DowAgroSciences 2016). 2,4-DB is not toxic to plants in the applied form, however upon foliar absorption, it is not as readily oxidised into the active 2,4-D form by legumes as it is in other broadleaf plants (Pillmoor & Gaunt 1981; Swarbrick & Mercado 1987). Susceptible plants will β-oxidise the butyric acid side chain into acetic acid, and active 2,4-D accumulates within the plant (Ketchersid *et al.* 1978). 2,4-D can bind to the TIR1 (transport inhibitor response 1) protein binding site in place of IAA, depressing gene expression through ubiquitination and degradation of transcription factors. This essentially prevents diffusion and degradation of IAA throughout the plant, which causes high levels of IAA to accumulate (Cobb & Reade 2010; Fedtke

1982). However, Evans *et al.* (1989) has observed that 2,4-DB often causes long term growth suppression in young, and established white and red clover plants, and tolerance in white clover has been found to increase as the plant develops from the seedling to 4th trifoliate leaf stage (Rolston 1987).

2.5.1.2 MCPB

MCPB (4-(4-chloro-2-methylphenoxy)butanoic acid) is also an auxin-type herbicide, out of patent, developed from the older herbicide formulation, MCPA (PubChem 2016d). Dow AgroSciences MCPB will be used in this study which contains 385 g/L MCPB as the sodium salt in the form of a soluble concentrate. MCPB is β-oxidised into the active form MCPA in the same mechanism as 2,4-DB, at which point it undergoes the same molecular action (Cobb & Reade 2010; Pillmoor & Gaunt 1981; Swarbrick & Mercado 1987). In legumes, MCPB is β-oxidised to MCPA, then hydroxylated to HMCPA, which undergoes conjugation to form a HMCPA glucose conjugate which is entirely non-toxic (Cobb & Reade 2010; Swarbrick & Mercado 1987).

2.5.2 Inhibition of photosynthesis

Photosynthesis is the conversion of solar energy (intercepted radiation) into chemical energy (ATP). The photosynthetic conversion process occurs in chloroplasts, and involves the oxidisation of water within the thylakoid membrane to protons, electrons, and oxygen. Protons and electrons are transported along the thylakoid membrane, generating ATP and reduced $NADP^+$, which is subsequently used to reduce CO_2 to carbohydrate in the Calvin cycle via enzymatic pathways in the stroma of the chloroplast (Taiz & Zeiger 2010).

All herbicides which act in photosystem disruption serve to kill target plants through photoperoxidation of the thylakoid membranes and other membrane lipids (Cobb & Reade 2010). A primary target for herbicide compounds disrupting the PSII pathway is the Q_B plastoquinone-binding niche in the D1 region (Trebst *et al.* 2002). Once the compound has bound to the central binding niche, plant metabolism at the photosynthetic electron flow level is blocked. Singlet oxygen molecules ($^1O_2$) are produced in the vicinity of the PSII reaction centre which react directly with the D1 protein causing photodamage which leads to photoinhibiton (Fedtke & Duke 2004; Trebst *et al.* 2002). Normal photodamage and inhibition is mediated via the photosynthetic repair cycle. However the binding of the active ingredient to Q_B prevents this repair cycle, triggering physiological cascades which destroy plant membranes and tissues through overproduction of membrane fatty acid peroxidation products, ethane, and malon dialdehyde (Melis 1999; Taiz & Zeiger 2010). With optimal herbicide activity, these products are present within the plant at detectable levels within 1-2 days. However with lower temperatures and reduced sunlight, the processes can take longer to occur (Fedtke 1982). Cold temperatures can affect the efficacy of photosystem II herbicide treatments, as photosynthetic processes rely on the occurrence of active growth within the plant, and decreases in temperature are correlated with decreases in membrane fluidity (Taiz & Zeiger 2010). PSII inhibition has been found to occur more rapidly *in vivo* in temperatures between 10 and 25˚C. However loss of efficacy below 10˚C is dependent on the active ingredient binding site, and recovery rates from low temperatures occur rapidly. PSII functionality is completely lost at 0˚C (Chow *et al.* 1989), and any herbicide treatment applied at temperatures below this would have no effect.

Visible symptoms following herbicide application in optimal conditions (full sunlight with a 100% electron flow inhibition success) will present as wilting of leaf tissue within hours, followed by a dark brown desiccation within days. When conditions are less optimal and sunlight is more limited, inhibition will occur at a slower pace, with leaf tissue bleaching from a light green to yellow, eventually turning white before becoming necrotic (Fedtke 1982; Fedtke & Duke 2004).

2.5.2.1 Bentazone

Bentazone is a herbicide formulated and marketed by BASF with the active ingredient bentazone (or bentazon) (3-Isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide) (PubChem 2016b). Basagran contains 480 g/L bentazone in the form of a soluble concentrate (BASF 2012a). Bentazone is a thiadiazine compound, classified as a miscellaneous photosystem II (PSII) inhibition herbicide (Cobb & Reade 2010). Bentazone has no residual soil activity, and is often mixed with other post emergence broadleaf herbicides to increase the weed spectrum controlled. Bentazone has been found to be one of the safer herbicides for use on legumes (Evers *et al.* 1993).

Bentazone (a.i. 0.8 kg/ha) treated 'Clare' subterranean clover showed 0-15% visible herbicide damage, and yields 450 – 1420 kg DM/ha, an average 53% increase compared with the unsprayed, unweeded control over a three year trial from 1986-1989 (Evers *et al.* 1993). In the same trial, bentazone (a.i. 1.7 kg/ha) caused 0-8% visible injury to the 'Clare' subterranean clover, and yields were 380-1250 kg DM/ha, an average 19% increase compared to the unsprayed unweeded control over the three years. Variations in yields over the years were attributed to a later application (20 days before first harvest, instead of 30) causing prolonged yield reductions, and variations in plant development stage, ranging from 4-12 trifoliate leaves depending on the year.

2.5.3 Inhibition of EPSP synthase

Glyphosate (N-phosphonomethyl glycine) (PubChem 2016c), was discovered by Monsanto in 1971 and patented in 1974 for use as a non-selective herbicide (Cobb & Reade 2010). Its action within the 5-enoylpyruvalshikimate-3-phosphate synthase (EPSPS) pathway was characterised by Jaworski (1972), with the EPSPS enzyme being formally identified by Steinrücken and Amrhein eight years later

(Steinrücken & Amrhein 1980). EPSPS is a plant-specific enzyme involved in the mass biosynthesis of tryptophan, phenylalanine and tyrosine, aromatic amino acids, as well as secondary metabolite phenylpropanoid metabolism and production of lignin, phytoalexins, ultraviolet light protectants, and anthocyanins (Fedtke & Duke 2004; Herrmann & Weaver 1999; Steinrücken & Amrhein 1980). Approximately 20% of carbon fixed by green plants is routed through the shikimate pathway, which produces a large number of essential compounds such as vitamins, alkaloids, and flavonoids (Cobb & Reade 2010; Taiz & Zeiger 2010).

EPSPS originates in the cytoplasm, and is imported into the chloroplast as a precursor. EPSPS must first react with shikimate-3-phosphate, then with phosphoenol pyruvate (PEP) in a reversible enzyme reaction (Herrmann & Weaver 1999; Steinrücken & Amrhein 1980). Plants will rapidly accumulate the precursor molecule shikimate upon treatment with glyphosate. Shikimate accumulation is indicative of dephosphorylation of the immediate precursor molecule shikimate-3-phosphate. This dephosphorylation and shikimate accumulation is a rapid, energy heavy process for herbicide treated plants. Shikimate accumulation can account for up to 16% of dry weight in the sink tissues where glyphosate accumulates – the roots, rhizomes and tubers. (Fedtke & Duke 2004).

Approximately 20 hours post treatment, chloroplasts in young leaf tissue have swollen, rupturing within four days (Cobb & Reade 2010). Visibly, glyphosate treated plants will cease growing within hours to days, depending on the rate of metabolic action, as this is the time taken to deplete the available aromatic acid stores within the plant. Once growth has ceased, leaves will begin to yellow and undergo chlorosis, with chlorophyll breakdown occurring some days later, and plants dying and decaying (Fedtke & Duke 2004).

2.5.3.1 Glyphosate

Glyphosate (N-phosphonomethyl glycine) is a major non-selective, post-emergence herbicide used when total vegetation control is desired with a low residual soil activity (Cobb & Reade 2010; Novachem 2016).

WeedMaster G360 contains the active ingredient 360 g/L glyphosate as the isopropylamine salt in the form of a soluble concentrate, formulated and distributed in New Zealand by Nufarm Limited (PubChem 2016d). It is safe for use on white clover at rates of 0.6 and 0.8 L/ha (Riffkin *et al.* 2005), but is recommended as a weedkiller for subterranean clover. This action will be confirmed in this research.

'Roundup', with the active ingredient glyphosate 490 g/L applied to subterranean clover in 2001 at Grogan, New South Wales, Australia at a rate of 0.6 L/ha showed reductions in herbage yields relative to unsprayed controls of 70-100% for cultivars 'Urana', 'Napier', 'Coolamon', 'Dalkeith', 'York', 'Gosse', and ‘Riverina’. The following year, in 2002 at Uranquinty in NSW, Australia, yields were reduced again, from 58-81% in cultivars ‘Urana’, ‘Napier’, ‘Coolamon’, ‘Dalkeith’, However yields of ‘York’, ‘Gosse’, and ‘Riverina’ were not different from the unsprayed control yields, with 200-800 kg DM/ha (Sandral & Dear 2005).

2.5.4 Acetolactate synthase inhibition

The inhibition of the capability of a plant to synthesise its own amino acids has become a major target in herbicide development (Cobb & Reade 2010). Such herbicides have low levels of mammalian toxicity, as mammals are incapable of synthesising the targeted amino acids (histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, phenylalanine and valine – the essential amino acids). All nitrogen in amino acid synthesis is derived from glutamate in the chloroplast stroma (combined action of glutamine + glutamate synthase) (Taiz & Zeiger 2010).

The enzyme which is inhibited by these herbicides has two names: acetolactate synthase (ALS) and acetohydroxyacid synthase (AHAS), as it is responsible for the catalysis of the first step in a biosynthetic pathway to produce either 2-acetolactate or 2-acetohydroxybutyrate (Fedtke & Duke 2004). This pathway can lead to the production of either valine/leucine as an endpoint, or isoleucine. These reactions are condensation reactions. ALS is a thiaminepyrophosphate (TPP) enzyme which produces a TPP-hydroxyethyl intermediate once CO_2 is limiting. It can be measured by testing acetolactate levels, hence the common abbreviation ALS.

Acetolactate synthase ALS (or acetohydroxy acid synthase AHAS) inhibition can be due to the activity of any one of the five new herbicide classes developed since the 1980s: Sulfonylureas, Imidazolinones (imazethapyr), Triazolopyrimidines (flumetsulam), Pyrimidinyl(thio)benzoates, and Sulfonyl amino carbonyl-triazolinones. These herbicides are potent, selective, broad-spectrum plant growth inhibitors, applied at low rates (g/ha rather than kg/ha), with increasing potency over time (Cobb & Reade 2010; Fedtke & Duke 2004). ALS inhibitors are capable of controlling a wide spectrum of both annual and perennial grass and broadleaf weeds in low dosages. Products are foliar and soil-active (Cobb & Reade 2010).

The branched chain amino acids valine, leucine, and isoleucine are derived from the alpha-keto acid pyruvate, but isoleucine also requires threonine in its synthesis (Azevedo *et al.* 1997). There are eight enzymes involved in the catalysis reactions of the precursor molecules pyruvate and threonine into these amino acids. ALS/AHAS and the next two enzymes in the pathway serve both production pathways (valine/leucine and isoleucine) (Taiz & Zeiger 2010). During enzyme catalysis, it is thought that the herbicide non-competitively inhibits the condensation reaction of activated aldehyde (the

TPP-bound decarboxylation product of the first pyruvate) with either 2-oxobutyrate or the second pyruvate molecule (Schloss 1990).

Whilst it is not fully understood how plants exposed to ALS inhibitors die, there are many diverse repercussions which can be observed at the physiological plant level post-treatment (Cobb & Reade 2010). Genomic results have suggested that accumulation of the intermediate products 2-oxobutyrate and 2-aminobutyrate play an important role in the toxic effects of ALS inhibitors (Jia *et al.* 2000). Free amino acid reserves of valine, leucine and isoleucine are temporarily depleted, until meristem tissue can reverse the inhibition, and other areas can switch to recycling branched chain amino acids through protein degradation. However the temporary depletion has further regulatory feedback effects which dramatically affect availability within other free amino acid pools (Brown *et al.* 2012; Cobb & Reade 2010). Within 5-7 hours of application, cellular regeneration cycles are inhibited in the growth, DNA synthesis, and mitosis stages. Protein turnover and soluble amino acid levels increase whilst soluble protein levels decrease. Phloem loading and phloem transport are strongly inhibited, which increases the availability of free reducing soluble sugars and sucrose, and respiratory pathways are inhibited (Fedtke & Duke 2004; Schloss 1990).

A common observation post ALS inhibitor application is the rapid and potent inhibition of cell division, causing cessation of growth at the meristem, and young roots and leaves are unable to elongate within a few hours of application (Tranel & Wright 2002). This causes plant tissue, particularly the leaves, to lose turgor and wilt. Within several days, yellow, pink and purple colouration symptoms can appear as a result of stress-induced anthocyanin accumulation (Fedtke & Duke 2004). Under optimal growth conditions, plant death may occur within 10 days of application. However if conditions do not allow ideal growth, symptom development is slow, and whole plant death can take up to two months (Cobb & Reade 2010). Symptom responses to ALS inhibiting-herbicides are relatively unspecific, and as a whole present as a reduction in growth rate and loss in plant vitality (Fedtke & Duke 2004). It is predicted that such potent ALS inhibitors with residual soil activity are likely to lead to an increase in resistant weed development (Cobb & Reade 2010).

2.5.4.1 Imazethapyr

Spinnaker is a herbicide formulated and marketed by BASF with the active ingredient imazethapyr (5-ethyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid) (PubChem 2016e), from the Imidazolinone class of ALS inhibiting herbicides. Imazethapyr has a 42 day stock withholding period (BASF 2011).

Imazethapyr has been shown to cause increases in leucine and isoleucine in tolerant plants, with only valine decreases apparent in the available amino acid pools post application (Royuela *et al.* 2000).

Carbohydrate accumulation in leaves has been repeatedly reported after treatment with ALS inhibitors (Roberts 2000; Royuela *et al.* 2000; Zabalza *et al.* 2004), and it is suggested that the carbohydrate accumulation in leaves could be a result of a failure in the phloem transport system. This would explain the breakdown of plant carbohydrate transport and may be part of the plant death mechanism, as meristematic tissues would be in a carbon-starved state (Zabalza *et al.* 2004). It was also found that the roots of imazethapyr-treated plants failed to appropriately use carbohydrates as a result of a reduced metabolic rate, causing the carbohydrate accumulation in leaves from decreased sink (root metabolism) strength (Zabalza *et al.* 2004).

'Clare' subterranean clover treated with imazethapyr (700 g ai/ha) had a visible injury of 13-22%, and a 63% reduction in total clover dry matter in the first year of trials, and 0-7% visible injury, with 4% reduction in total clover dry matter yields in the second year following reapplication (Evers et al., 1993). Dear and Sandral (1999) found application of imazethapyr (240 g/L imazethapyr) to 'Karridale' and 'Trikkala' subterranean clover swards caused no leaf burn effects in plants at either the high (72 g ai/ha) or low (43.2 g ai/ha) application rate. Imazethapyr application (72 g ai/ha) on 8 July 1992 caused yield reductions of 21% to 'Trikkala' and 28% to 'Karridale', compared to the unsprayed, handweeded controls at 30 days post application. These decreased to reductions of 3% for 'Trikkala' and 7% for 'Karridale' by 90 days after application. Decreases in yield observed in the low rate treatment were similar at both 30 and 90 days post application. The following year, treated 'Karridale' and 'Trikkala' had observed yield increases of 13%, and 7% respectively at 90 days post application relative to unsprayed controls. In a separate experiment, imazethapyr applied to 'Antas' subterranean clover caused only a 2% yield reduction 50 days post application relative to unsprayed, handweeded controls. The following year, imazethapyr treated 'Antas' had a 48% reduction at 25 days post application, however significant recovery occurred to produce only a 7% yield reduction at 50 days post application compared with the controls (Sandral & Dear 2005).

Imazethapyr applied to subterranean clover at a rate of 72 g ai/ha in 2001 at Grogan, New South Wales, Australia showed variable reductions in herbage yields relative to unsprayed controls. 'Dalkeith', 'Riverina', 'Gosse', 'Coolamon', 'Urana', and 'Napier', with clover yield reductions of 0-49% were not different from the unsprayed control yields of 700-1350 kg/ha while the 76% reduction in yield observed in 'York', was different to the unsprayed clover yield of 1300 kg/ha. The following year, in 2002 at Uranquinty in NSW, Australia, yields were reduced again, but were dissimilar to the previous year; 'Napier', 'Urana', and 'Dalkeith', were no different to the unsprayed clover yields of 300-650 kg/ha. 'Riverina', 'York', 'Gosse', and 'Coolamon' had yield decreases of 57-93%, and were different from the unsprayed clover yields of 200-800 kg/ha (Sandral & Dear 2005).

In Australia, the recommended ALS inhibiting herbicide for use on subterranean clover is Raptor® which contains 700 g/L of the active ingredient imazamox. In trials in 2001 at Grogan, NSW with Raptor® applied at 20 g ai/ha, 'Urana', 'Gosse', 'Dalkeith', 'Napier', 'Coolamon' and 'York' showed no effect on clover yields compared to unsprayed controls with 700-1350 kg/ha. The following year, at Uranquinty in NSW, yield reductions for cultivars were no different to the unsprayed control yields of 200-800 kg/ha (Sandral & Dear 2005). Unfortunately, Raptor®, developed and marketed by BASF will not be licensed for use in New Zealand (G Hagerty 2016. Pers comm, 27 January), therefore it will not be included in this study.

2.5.4.2 Flumetsulam

Headstart is a flumetsulam (N-(2,6-difluorophenyl)-5-methyl-[1,2,4]triazolo[1,5-a]pyrimidine-2-sulfonamide) (PubChem 2016g) herbicide, of the triazolopyrimidine family, with ALS-inhibitory action. This class of herbicides was originally developed as bioisosteric analogs of the sulfonylureas (Brown *et al.* 2012). This means they have similar modes of action, but minor alterations in the chemical structures and modes of *in vitro* activity which lead to differences in selectivity against species and differences in physiological symptoms. Flumetsulam contains 50 g/L flumetsulam in the form of an oil dispersion, and is formulated and distributed in New Zealand by Zelam Limited (Zelam 2016). Flumetsulam is currently the only herbicide recommended for use on subterranean clover in the Novachem New Zealand Agrichemical Database (Novachem 2016).

Application of Broadstrike® herbicide (a.i. flumetsulam 800 g/kg) applied at 25 g ai/ha to 'Antas' in Australia in 1999 showed no reduction in clover herbage yield at 50 days post application compared with an unsprayed, handweeded control yield of 9900 kg/ha. The following year, there was again no difference from the unsprayed handweeded control yield of 5300 kg/ha by day 50 (Sandral & Dear 2005).

2.5.5 Inhibition of protoporphyrinogen oxidase

Chlorophyll is the primary pigment involved in photosynthesis. It is located within the reaction centres, playing a pivotal part in the relocation of electrons from a low energy (H_2O) to high energy ($NADP^+$) state (Taiz & Zeiger 2010). Protoporphyrinogen oxidase (PPO) is part of a multienzyme complex involved in the synthesis of the tetrapyrrole structures chlorophyll, heme, and cytochrome. PPO is responsible for the oxidation of protoporphyrinogen IX to protoporphyrin IX via molecular oxygen, producing H_2O_2 as a by-product. When PPO is inhibited, the protoporphyrinogen IX substrate is released from the mulitenzyme complex and is free to spontaneously react with molecular oxygen, forming protoporphyrin IX, outside of the PPO pathway (Grossmann *et al.* 2001).

The free protoporphyrin IX generated in the spontaneous oxidation of protoporhyrinogen IX is incapable of reacting with the magnesium-chelatase, a three-component enzyme that catalyses the insertion of Mg^{2+} into protoporphyrin IX to form either Fe- or Mg-chelates. This causes a loss in the regulation of chlorophyll synthesis via protoporphyrinogen IX, due to the loss of the tetrapyrrole precursor (Mg-chelate). Protoporphyrin IX is synthesized in large amounts, and in dark conditions it can benignly accumulate in plant tissue. In light conditions it acts as a photosensitizer, and transfers its excitation energy to molecular oxygen, forming singlet oxygen ($O_2(^1\Delta_g)$) (Fedtke & Duke, 2005; Grossmann et al., 2010; Lee et al., 1993). Singlet oxygen is highly reactive, and interacts with unsaturated membrane lipids, initiating peroxidative chain reactions, which have the downstream effect of oxidative destruction of membranes and other cellular constituents (Cobb & Reade, 2010; Fedtke & Duke, 2005).

As such, the major destructive action of a PPO-inhibiting herbicide is through excessive accumulation of activated singlet oxygen species (Lee et al., 1993). Within 1-3 hours of application, photosynthetic activity will cease, preventing CO_2 fixation, and this is accompanied by swelling of chloroplasts and damage to membranes. Once membrane damage has occurred, cells will lose turgor, causing cellular compounds to leak into the apoplast (Grossmann et al., 2010). Both chloroplasts and mitochondria (cytochromes) are affected as both contain PPO. However the effects in chloroplasts occur twice as fast, due to higher maximal synthesis rates. This means damage occurs extremely rapidly in leaves, which are exposed, while it progresses at a much slower rate in other tissues (Fedtke & Duke, 2005). Over time, the accumulation of lipophilic vesicles within the cytoplasm produces ethane, pentane and malondialdehyde as by-products of membrane lipid breakdown. Carotenoids, which can quench singlet oxygen to an extent are destroyed once overloaded, and chlorophylls, which are protected by the carotenoids and α-tocopherol follow 1-2 days later (Fedtke & Duke, 2005; Grossmann et al., 2010). ACC synthase initiates ethylene production as part of the stress response, and excessive ethylene accumulations often lead to production of abscisic acid, followed by leaf abscission (Cobb & Reade, 2010; Fedtke & Duke, 2005). Other stress responses are activated as a result of prolonged sublethal levels of singlet oxygen, initiating synthesis of phytoalexins (Cobb & Reade, 2010).

PPO-inhibiting herbicides primarily affect dicotyledonous species (Fedtke & Duke, 2005). The contact activity of peroxidising herbicides often leads to poor selectivity (Cobb & Reade, 2010). Visibly, plants affected by a PPO inhibiting herbicide will develop phytotoxicity symptoms within a few hours to days. Reactions will occur faster in full sunlight as a result of the photosensitivity of protoporhyrin IX. Visible symptoms begin with wilting, and browning of tissue, followed by necrosis in rapid-action conditions, or leaves lightening to a light green colour, followed by chlorosis and eventual necrosis in slower-action conditions (reduced growth rates) (Cobb & Reade, 2010; Fedtke & Duke, 2005).

2.5.5.1 *Saflufenacil*

Sharpen is a herbicide formulated and marketed by BASF New Zealand Limited with 700 g/kg saflufenacil ((2 - chloro - 4 - fluoro - 5- [3-methyl - 2,6 – dioxo – 4 - (trifluoromethyl) pyrimidin-1-yl] – N - [methyl (propan-2-yl) sulfamoyl] benzamide) (PubChem, 2016h) as the active ingredient in the form of a water-dispersible granule (Saflufenacil Specimen Label 2015). Saflufenacil is a pyrimidinedione compound, which works by inhibiting protoporphyrinogen IX oxidase (PPO). It was recommended safe for use on subterranean clover and was therefore tested in this research (BASF, G Hagerty, pers comm., 27 January 2016).

2.5.6 Mixed action herbicides

2.5.6.1 *Bentazone + MCPB*

Pulsar is an MCPB + bentazone mix herbicide marketed by BASF New Zealand Limited. It combines the auxin-type action of MCPB with the photosystem II photosynthesis inhibition of bentazone for quicker senescence of target plants. Pulsar contains 200 g/L MCPB + 200 g/L bentazone as the sodium salts in the form of a soluble concentrate (BASF 2012c). Visible symptoms following treatment should be a combination of those seen in plants treated with MCPB or bentazone.

2.5.6.2 *Bromoxynil + diflufenican*

Jaguar is a herbicide formulated and marketed by Bayer CropScience, containing 250 g/L bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) (PubChem 2004) + 25 g/L diflufenican(N-(2,4-difluorophenyl)-2-[3-(trifluoromethyl)phenoxy]pyridine-3-carboxamide) (PubChem 2016f). Bromoxynil is a nitrile compound; a photosystem II inhibitor of plant growth with a mode of action similar to that of bentazone (Cobb & Reade 2010). Diflufenican is a triazolopyrimidine compound which inhibits chloroplast isopropenoid biosynthesis through inhibition of phytoene desaturase (PDS) (Cobb & Reade 2010). Chloroplast isopropenoid-inhibiting herbicides induce growth of new leaves with white tissue which lacks chloroplasts. Diflufenican's inhibiton of PDS causes an accumulation of phytoene, preventing the complex isopropenoid network pathways from proceeding. As PDS is the first step in the isopropenoid network, it is complete inhibition, ultimately preventing the activity of the carotenoid biosynthesis pathway (Fedtke & Duke 2004). Physiological treatment effects are triggered through exposure to excess light, which cannot be properly used by the plant (Cobb & Reade 2010).

Visibly, under optimal conditions, treated plants will produce plant tissue which is completely white, but otherwise unaffected. Within 1-3 days, this white tissue will begin to wilt, then die from the lack of chloroplasts promoting photosynthetic action. In conditions with less available light, chloroplasts can develop and the plant produces green tissue due to lower light requirements for chlorophyll biosynthesis, and chlorophyll light protection by the carotenoids is not required. Upon exposure to strong light, singlet oxygen molecules are produced, and cell constituents are rapidly peroxidised, with plant tissue decaying, visibly turning brown and necrotic (Fedtke & Duke 2004).

Weeds which are susceptible will die relatively rapidly as a result of a build-up within the plant of molecules which destroy cellular membranes (Cobb & Reade 2010). Diflufenican is persistent within the soil, and germinating weeds will lack photoprotective carotenoids, so lipid peroxidation will occur following germination, bleaching the weeds which rapidly die (Cobb & Reade 2010; Fedtke & Duke 2004).

Dear and Sandral (1999) found that bromoxynil + diflufenican was the most phytotoxic to pasture seedlings of those herbicides evaluated in a study, causing severe leaf burn and a depression in herbage biomass. However, they suggested that this was justified through the increased cruciferous weed control attained. When applied to 'Trikkala' in July 1992 at a rate of 0.5 L/ha, yield decreases of 26% at 90 days after application compared to the unsprayed handweeded control, which yielded 9800 kg/ha clover. When treated in August 1993, treatment yields at 90 days after application were not different to the unsprayed handweeded control with 5900 kg/ha clover. When applied at a rate of 1.0 L/ha, 'Trikkala' had yield reductions of 31-33% over both years 90 days after application, compared to the unsprayed controls. 'Karridale' treated at both rates had yield reductions of 21-36% at 90 days after application over the two years of the trial, different to the unsprayed handweeded control yields of 6100-10400 kg/ha.

In a later study in 1999, 'Jaguar' applied at 750 ml/ha to 'Antas' showed no difference in yield to the unsprayed handweeded control at 25 or 50 days after application, with yields of 1900 kg/ha and 9910 kg/ha respectively. The following year, at 25 days after application, yields were reduced by 75% compared to the unsprayed control with 3950 kg/ha, which then recovered to be no different to the unsprayed control with 5330 kg/ha at 50 days after application (Sandral & Dear, 2005).

2.6 Phytotoxicity of herbicides in subterranean clover

Subterranean clover tolerance to herbicide treatment can differ from year to year depending on the season; the rainfall, temperature, available soil moisture, and when that rainfall occurs (Dear *et al.* 1992). Cultivars showed reduced tolerance to a grass killer, simazine, in a wetter year, with a rainfall of 704 mm, and an average 65% reduction in yield for all cultivars. Relative tolerance doubled in the following year, with 35% yield reductions, with a rainfall of 564 mm. Despite the environmental variance, the yields of the unsprayed controls remained the same, at 4.64 t/ha in 1989, and 4.70 t/ha in 1990. 'Trikkala' showed consistent herbicide tolerance, while 'Karridale' showed consistent herbicide sensitivity (Dear *et al.* 1992). As a *yanninicum* adapted to more waterlogged soils (Dear &

Sandral 1997), the response of 'Trikkala' in this situation was likely due to environmental adaptation, but mechanisms of tolerance were not discussed. A two year experiment in Wagga Wagga, Australia, found that that subterranean clover cultivars show differential tolerance to herbicide application, with reductions in herbage yield of 16-71% compared to 1480-2560 kg/ha in an unsprayed, handweeded control. 'Seaton Park' showed high sensitivity to MCPA + terbutryn, MCPA + diuron, and bromoxynil. 'Karridale' showed high sensitivity to MCPA treatment. 'Trikkala' was again the most herbicide tolerant cultivar to 2,4-DB, MCPA , bromoxynil, MCPA + terbutryn, and MCPA + diuron (Dear *et al.* 1995). 'Trikkala' is a *yanninicum* cultivar, however there was no available information on its reason for heightened tolerance, and is just considered a more tolerant cultivar, while 'Karridale', a *subterraneum* cultivar, is widely considered herbicide sensitive.

Dear and Sandral (1999) concluded from their assessment of phytotoxicity of a range of herbicides (pyridate, imazethapyr and bromoxynil + diflufenican) to seedling subterranean clover and lucerne pasture mixes that even if the subterranean clover suffers significant phytotoxicity damage, herbicide use was justified. This was from increased subterranean clover seed set, and increased lucerne yields later in the season due to weed suppression. They concluded that all herbicides tested could be applied to subterranean clover – lucerne mixes, despite yield losses of up to 21% (compared to a weed free, unsprayed sward), as the components had the capacity to recover from the phytotoxicity damage (Dear & Sandral 1999).

2.7 Conclusions

Based on this literature review, the following conclusions can be drawn:

While it is widely accepted that there is a cultivar*herbicide interaction for subterranean clover herbicide tolerance, there is no discussion of the potential causes behind this difference in tolerance available in the literature. There is an abundance of available data on herbicide tolerance of subterranean clover in Australia, but this is not fully applicable to the New Zealand environment, and the smaller market means reduced herbicide availability. Yield reductions in subterranean clover cultivars as a result of herbicide application can vary from year to year depending on the season, likely in response to temperature and rainfall fluctuations. In most cases, herbicide application caused a check to subterranean clover growth, which recovered to benefit from chemical weed control measures. The more temperate climate of New Zealand makes the transfer of this knowledge less straightforward. While there is a range of commercially available herbicide options for white clover in New Zealand, only one, flumetsulam, is registered for use on subterranean clover. Despite its registered status, there is no published information on how it affects subterranean clover in New Zealand. Guidelines for application times are broad and based off white clover. Currently, there is no available field data for herbicide effects on recent New Zealand cultivars or the importance of development stage at application.

The majority of current research on subterranean clover tolerance is for pure swards, grown as cover crops and for seed rather than in intensive pasture systems. As a result, there is little available on how the herbicides work in true field scenarios, in comparison to unsprayed unweeded controls. And no available research on how subterranean clover establishment is affected by weeds. As a result, there is no discussion of the effects of broadleaf herbicides to eliminate weeds can provide subterranean clover with an advantage, or if the potential reductions in subterranean clover content of the sward are not worth it. Further to this, there is no available information on how these herbicides work with subterranean clover-grass mixtures, and how to improve their establishment with the aid of herbicides.

There is also no discussion of the biochemical and molecular potential for different responses of subterranean clover cultivars to the different herbicide active ingredients. Furthermore, investigation into any potential for longer lasting effects of these herbicides could provide more avenues for further research once identified.

3 EXPERIMENT 1: RESPONSE OF 13 CLOVER CULTIVARS TO TWO COMMERCIAL HERBICIDES: IMAZETHAPYR AND SAFLUFENACIL

3.1 Introduction

Subterranean clover has been shown to have differential herbicide tolerance in Australian herbicide trials (Dear *et al.* 1992; Evans *et al.* 1989; Sandral & Dear 2005). However there is a lack of available literature on the efficacy of any herbicides on subterranean clover in New Zealand. We do not have the full range of cultivars commercially available in Australia, because we are reliant on seed companies, and availability of clean seed for import (Monks *et al.* 2016). This chapter assesses the relative herbicide tolerance of the 13 subterranean clover cultivars available in New Zealand at the time of sowing, as well as red, white, and balansa clover controls.

The most promising herbicide identified from the literature which has the potential for aiding establishment of subterranean clover by eliminating weeds was Raptor® (a.i. 700 g/kg imazamox), an ALS inhibiting herbicide which was also recommended by Australian pasture scientists (pers comm. Lindsay Bell February 2016). As Raptor® is not, and will not become commercialised in New Zealand, imazethapyr (a.i. 240 g/L imazethapyr), also an ALS inhibiting herbicide was identified to be the closest commercially available herbicide in New Zealand for investigation. Imazethapyr was also tank mixed with saflufenacil (a.i. 700 g/kg saflufenacil), a PPO inhibiting herbicide which is said to 'sharpen up' the action of the herbicide it is mixed with, to assess its selectivity on subterranean clover.

Results from this experiment will be used to achieve Objective 1 (Figure 1.1), evaluating the visible and physical differences in herbicide tolerance between cultivars, and identifying a potential explanation for further investigation.

3.2 Materials and methods

3.2.1 Germination tests

Germination tests were performed in laboratory conditions prior to establishment of any experiments to confirm seed viability (Table 3.1).

Table 3.1 50 seed weight, thousand seed weight, approximate seeds/m^2 for intended sowing rate used, germination number and germination percentage for all species and cultivars used in all experiments.

Cultivar	50 SW (g)	TSW (g)	Seeds/m^2	Germ #	Germ %
'Antas'	0.553	11.06	181	49	98
'Campeda'	0.382	7.64	262	49	98
'Coolamon'	0.283	5.66	353	47	94
'Denmark'	0.346	6.92	289	45	90
'Karridale'	0.339	6.78	295	48	96
'Leura'	0.395	7.9	253	47	94
'Monti'	0.489	9.78	204	40	80
'Narrikup'	0.458	9.16	218	45	90
'Napier coated'	1.243	24.86	80	45	90
'Rosabrook'	0.418	8.36	239	40	80
'Rosabrook' coated	0.789	15.78	127	42	84
'Trikkala'	0.422	8.44	237	40	80
'Woogenellup'	0.506	10.12	198	49	98
'Whatawhata'	0.401	8.02	1247	39	78
'Bolta' balansa clover	0.052	1.04	481	36	72
'Rossi' red clover	0.109	2.18	229	46	92
'Nomad' white clover	0.054	1.08	463	46	92
'Greenly II' Cocksfoot	0.051	1.02	196	36	72

3.2.2 Long-term meteorological conditions

Long-term mean (LTM) weather data for Lincoln, Canterbury from 1960 to 2015 was retrieved from the NIWA Cliflo database. Canterbury's climate is characterised as cool and temperate with a long term mean temperature of 11.5 °C, ranging from 16.7 °C in January to 6.1 °C in July (Table 3.1). Long-term annual average rainfall is 631 mm, spread evenly across the year but occurring as sporadic events after dry spells in any given year. Annual Penman potential evapotranspiration (EP) is 1086 mm which generally exceeds rainfall from September to April causing a potential long-term soil moisture deficit of 534 mm per year.

Table 3.2 Monthly long-term means (LTM) from 1960 to 2015 for mean (T_{mean}) air temperature, rainfall, and Penman potential evapotranspiration (PET). Taken from the CliFlo database, measured at the Broadfields Meteorological Station, Lincoln, Canterbury, New Zealand.

Month	T_{mean}(°C)	Rainfall(mm)	PET(mm)
Jan	16.7	48.2	157
Feb	16.5	42.4	126
Mar	14.8	52.9	103
Apr	12.0	55.5	65
May	9.2	56.8	45
Jun	6.6	61.8	33
Jul	6.1	61.7	36
Aug	7.3	62.1	52
Sep	9.4	39.8	75
Oct	11.4	47.2	110
Nov	13.2	50.2	133
Dec	15.2	52.6	152
Annual	11.5	631	1087

3.2.3 Iversen 1

Experiments 1 and 2 were sown on the 01 March 2016 at Iversen 1 (43°38'57.4"S 172°28'07.8"E, 11 m above sea level), at Lincoln University, Canterbury, New Zealand. The soil at the site is predominantly a Wakanui silt loam (Udic Ustochrept, USDA Soil taxonomy)(Cox 1978). These are deep, stoneless and poorly draining soils (Hewitt 2010). A soil sample was taken on 01 March 2016 to assess available soil nutrient levels (Table 3.3) and showed no deficiencies.

Table 3.3 Soil test (0 - 75 mm) results for Iversen 1, Lincoln University, and Ashley Dene C9BS, Springston, Canterbury, New Zealand on 01 March 2016.

Analysis	Iversen 1	Ashley Dene
pH	5.5	6.0
Olsen Phosphorus (mg/mL)	17	28
Potassisum (me/100 g)	1.03	1.95
Calcium (me/100 g)	6.9	9.9
Magnesium (me/100 g)	1.65	0.92
Sodium (me/100 g)	0.26	0.25
CEC (me/100 g)	15	18
Sulphate Sulphur (mg/kg)	9	30
Potentially available nitrogen (15 cm depth) (kg/ha)	135	111
Anaerobically mineralisable N µg/g	91	84

The experimental site received an accumulated rainfall of <350 mm over the experimental period, from February to October 2016. Average air temperature for the period was 11.7 ˚C, and average soil temperature was 11.3 ˚C (Figure 3.1).

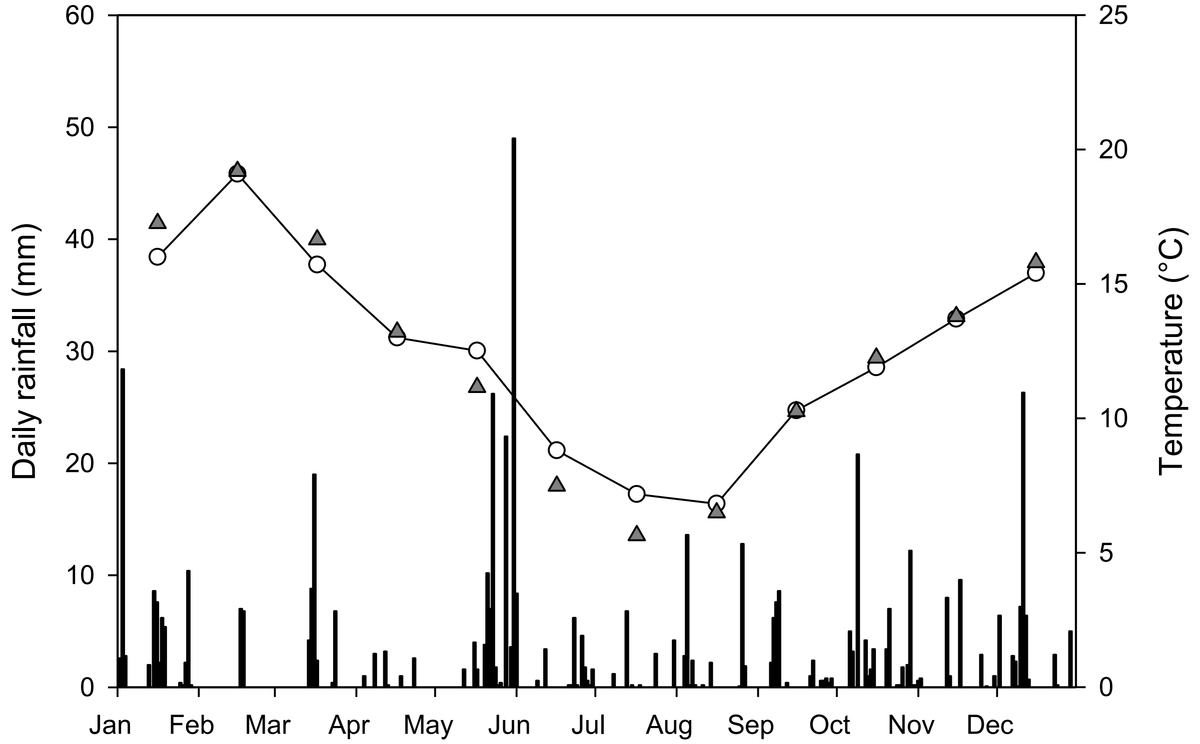

Figure 3.1 Daily rainfall (■) and mean monthly air (○) and 2 cm depth soil temperatures (▲) for January - December 2016 from Iversen 2 datalogger (temps) and Broadfields (rainfall) for Lincoln University. Rainfall is daily rainfall (mm); soil and air temperatures are monthly means.

3.2.4 Paddock history

The site was previously used as non-renewed pasture and grazed for the past 20 years (pers comm. Richard Lucas, 2016). Dominant resident weed species were identified prior to sowing, and throughout the experimental period (Table 3.4). Grass weeds were predominantly annual ryegrass (*Lolium multiflorium*), phalaris (*Phalaris aquatica*L.), and annual brome (*Bromus hordeaceus* L.).

Table 3.4 Common and botanical names of resident weed species at establishment of Experiments 1 and 2 in Iversen 1, Lincoln University, Canterbury, New Zealand.

Common name	Botanical name
Broad leaved dock	*Rumex obtusifolius* L.
Clustered dock	*R. conglomeratus* M.
Chickweed	*Stellaria media* L.
Dandelion	*Taraxacum officinale* L.
Fathen	*Chenopodium album* L.
Hawksbeard	*Crepis capillaris* L.
Mallow	*Malva sylvestris spp.* L.
Spurrey	*Spergula arvensis* L.
Twin cress	*Lepidium didymum* L.
Wireweed	*Polygonum aviculare* L.
Yarrow	*Achillea millefolium* L.

3.2.5 Site preparation

The site was sprayed on 22.02.2016 with 5 L/ha Buster herbicide (a.i. 200 g/l glufosinate-ammonium) in 200 L of water to kill the resident vegetation (Table 3.4). This created a bare seed bed for direct drilling.

3.2.6 Experimental design

Seventeen clover cultivars (Table 3.5) were direct drilled on the 01.03.2016 using a Flexi-seeder® drill at 15 cm drill width in 8 x 2.1 m cultivar plots in a complete randomized design, with three replicates. Subterranean clover sowing rates of 20 kg/ha were higher than commercially recommended to ensure establishment. 'Whatawhata' was sown at a rate of 100 kg/ha due to poor seed quality and failure to geminate well in germination tests. At the four trifoliate leaf stage, the two herbicide treatments, (Table 3.6) were applied in 2 m strips across plots, leaving a 2 m unsprayed, unweeded control strip in each plot, randomised for each replicate. This created a strip-split plot (criss-cross plot) design.

Table 3.5 Clover cultivars used in Experiment 1, subterranean clover subspecies, and sowing rate for Experiment 1 at Iversen 1, Lincoln University, Canterbury, New Zealand.

Species	Cultivar	Subspecies	Sowing rate (kg/ha)
T. subterraneum	'Antas'	*Brachycalycinum*	20
	'Campeda'	*Subterraneum*	20
	'Coolamon'	*Subterraneum*	20
	'Denmark'	*Subterraneum*	20
	'Karridale'	*Subterraneum*	20
	'Leura'	*Subterraneum*	20
	'Monti'	*Yanninicum*	20
	'Narrikup'	*Subterraneum*	20
	'Napier'	*Yanninicum*	20
	'Rosabrook'	*Subterraneum*	20
	'Rosabrook' coated	*Subterraneum*	20
	'Trikkala'	*Yanninicum*	20
	'Woogenellup'	*Subterraneum*	20
	'Whatawhata'	*Subterraneum*	100*
T. michelianum	'Bolta' Balansa Clover		10
T. pratense	'Rossi' Red Clover		10
T. repens	'Nomad' White Clover		10

**'Whatawhata' sown at a rate of 100 kg/ha due to the seed received (nucleus seeds; line AK1332) containing 30% broken seeds, likely damaged during harvest, as not a commercial line. Germination tests of undamaged seed showed 78% of seed germinated. In field germination could be significantly impacted, and lower than 50% (Table 3.1).*

3.2.7 Herbicide applications

Herbicide applications were carried out at 0900 on 7 April 2016 using a knapsack sprayer with a hand pump wand V110 04 AC nozzle with a spread of 1 metre and a walking speed of 5 km/h. Hebricides were applied in 200 L of water per hectare. Wind direction was a prevailing northerly, with an average speed 8-12 km/h for the duration of application. Average air temperature was 17.9 ˚C, and average soil temperature was 15.7 ˚C at 2 cm depth.

Table 3.6 Herbicide trade names, active ingredient, mode of action, and rate applied for Experiments 1 and 2. Alternative tradenames and commercial availability can be found in the Appendix (Table A.2).

Herbicide	Active Ingredient	Mode of Action	Product rate/ha
Imazethapyr	240 g/L imazethapyr	ALS inhibition	400 ml
Imazethapyr + Saflufenacil	240 g/L imazethapyr + 700 g/L saflufenacil	Increases rate of activity of mixed herbicide through PPO inhibition	400 ml + 25 g

3.2.7.1 Adjuvant

All herbicide treatments in this research were applied with the adjuvant Hasten™ (704 g/L ethyl and methyl esters of vegetable oil) at a rate of 500 ml per 100 L to eliminate active ingredient differences in permeability. This is a blend of esterified vegetable oil and non-ionic surfactants produced by BASF. Hasten™ is suggested to improve spray results by; increasing penetration through waxy cuticles, increasing wetting and spreading of spray droplets and reducing spray droplet evaporation rates (BASF 2012b).

3.2.8 Agronomic management

Irrigation was applied to all plots due to low April-March rainfall (Figure 3.1). Plots were irrigated with approximately 20 mm given wind variation on 13 May 2016, using a RM 540 gx pivot irrigator.

Plots were heavily grazed for a week with a mob of 100 ewe lambs from 12 September 2016 following the first harvest, then cut and carried using a Fieldmaster forage harvester and cage to clear the unpalatable weeds from the controls.

3.2.9 Measurements

3.2.9.1 Seedling establishment

Initial reductions in plant number (thinning) as a result of herbicide treatment within the first month of application can be tracked through plant counts (Kroschel 2001). During emergence, a representative 1 m section of drill row was permanently marked in the centre of each plot. Seedling counts of sown clover for each plot were carried out weekly until one month post herbicide application, when the seedling counts were stable.

3.2.9.2 Phytotoxicity assessment

All plots were visually scored on days 2, 5, 7, 10 and 14 after treatment application and weekly thereafter until the first harvest using the EWRS phytotoxicity scoring system (OEPP/EPPO 2014). Each treated plot was scored in its entirety, and designated a damage score relative to the unsprayed control plot of that cultivar, which has an EWRS damage score of 1.0, and no apparent phytotoxicity symptoms (Table 2.1).

3.2.9.3 Cultivar physiology

Cultivars were scored for petiole and leaf pubescence on 31 August 2016, using a system adapted from the subterranean clover in NSW - identification and use guide (Dear & Sandral 1997).

Table 3.7 Relative plant pubescence scoring system (Adapted from Dear and Sandral (1997))

Score	Pubescence
0	No hairs
1	Few hairs
2	Hairy
3	Very hairy

3.2.9.4 Herbage dry matter production

Phytotoxicity of a compound may only be apparent upon final harvest, with a quantitative or qualitative analysis of the yield and its components. This can be measured as dry matter in t/ha, percentage target plant vs. weed species in comparison to the control, and physiological symptoms not previously identified in the field (Kroschel 2001).

Herbage dry matter yield measurements were determined twice, on 07 September and 25 October 2016 with one cut of a representative 0.2m^2 quadrat from each plot. Samples were subsampled to approximately 50 g freshweight, then sorted into sown clover, broadleaf weeds, grass weeds, and dead matter. Both the subterraneansample and remainder were dried for a minimum 48 hours at 65°C before weighing.

Plots treated with Spinnaker + Sharpen were bare, with an EWRS score of 9.0 at the time of the first harvest. They were eliminated from physical harvesting, and recorded as a negative control.

'Trikkala', Balansa clover, 'Campeda', 'Monti', 'Napier' coated, and 'Rosabrook' cultivars were excluded from the second harvest as they were considered not tolerant of either herbicide based on results from the first harvest.

3.2.10 Statistical analysis

Statistical analyses were carried out in Genstat v. 16.1 Figures were made in SigmaPlot v. 11.

Results were analysed using a two-way split-strip plot ANOVAs of cultivar by treatment, with treatment as rows and cultivar as columns. Means separations were carried out using Fisher's unprotected Least Significant Difference tests at $\alpha = 0.05$. Repeated measures ANOVAs of seedling counts and EWRS scores were performed to analyse size of changes over time. Imazethapyr + saflufenacil was excluded from yield analyses as there was no surviving clover or broadleaf weeds in any of the plots.

Regression analyses of annual plant pubescent ratings for with EWRS scores; and EWRS scores with harvest yields were performed to identify correlations.

3.3 Results

3.3.1 Seedling establishment

The 'Whatawhata' line had a higher (P<0.001) number of seedlings per m^2 than all other cultivars due to its higher sowing density (Figure 3.2, Table 3.1, and Table 3.5). 'Rosabrook', 'Monti', 'Rosabrook' coated and 'Napier' coated line had the lowest (P<0.001) number of seedlings.

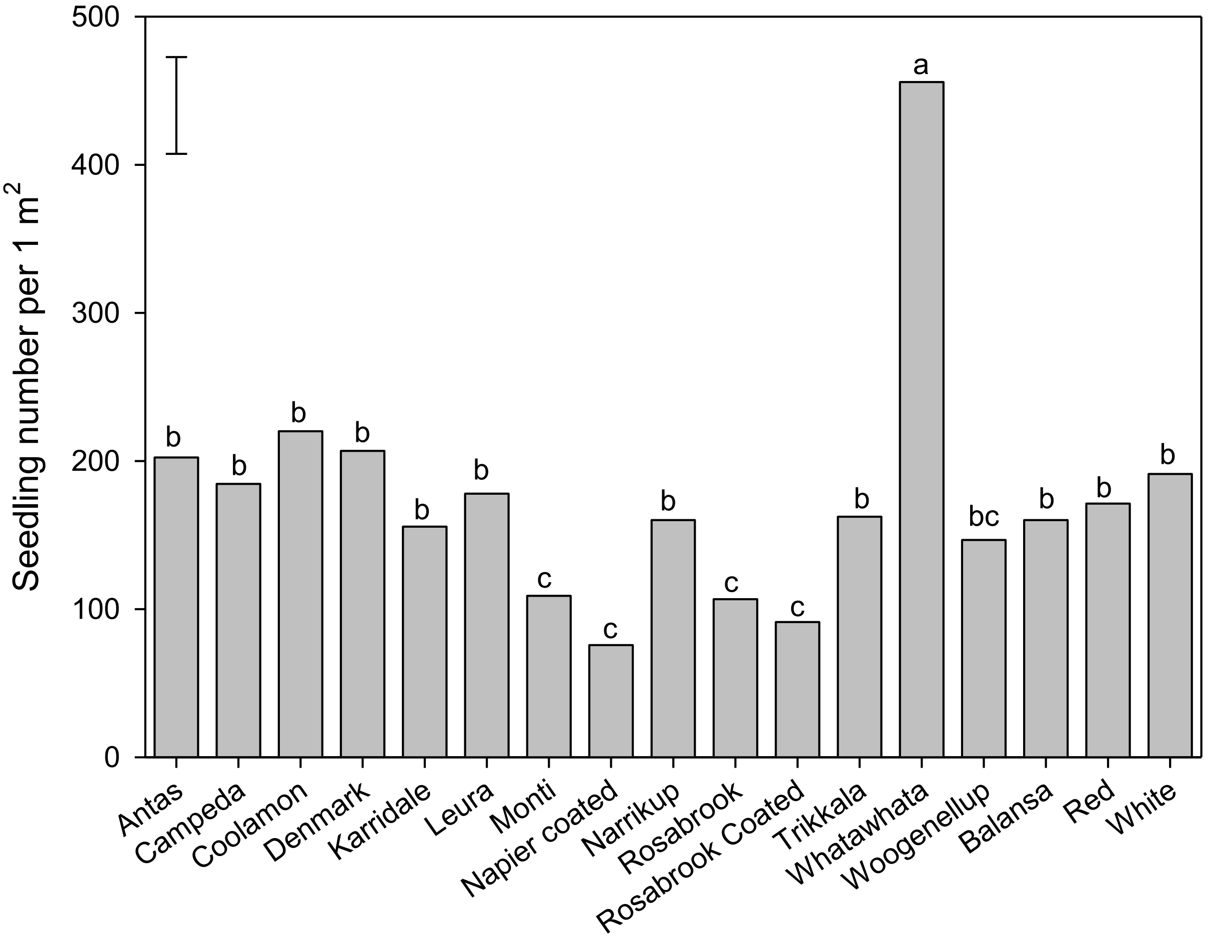

Figure 3.2 Seedling number per 1 m^2 for all clover cultivars on 6 April 2016 prior to herbicide application at Iversen 1, Lincoln University, Canterbury, New Zealand. Error bar is LSD for cultivar differences. Bars with a letter in common are not significantly different at α=0.05.

The number of seedlings per 1 m^2 changed over 28 days (P<0.001) from an average 200 seedlings per m^2. Imazethapyr treated plots showed no difference in seedling number to unsprayed unweeded controls by 28 DAA, with all cultivars the same as their controls. Imazethapyr + saflufenacil treated plots had fewer (P<0.001) seedlings than both the unsprayed unweeded controls, and the imazethapyr treated plots. Unsprayed unweeded controls and imazethapyr treated plots had 100-130 seedlings per m^2 by 28 DAA, while imazethapyr + saflufenacil treated plots had 55 seedlings per 1 m^2 (Figure 3.3). This rapid response to the imazethapyr + saflufenacil treatment was visible within 7 days of treatment application, where seedling numbers had dropped to 110 seedlings per m^2 (Figure 3.3 C, and Plate 3.1). In comparison, the unsprayed unweeded control and imazethapyr showed no reduction at 7 DAA compared with immediately prior to application, with 170-220 seedlings per m^2 (Figure 3.3 A, and B and Plate 3.1).

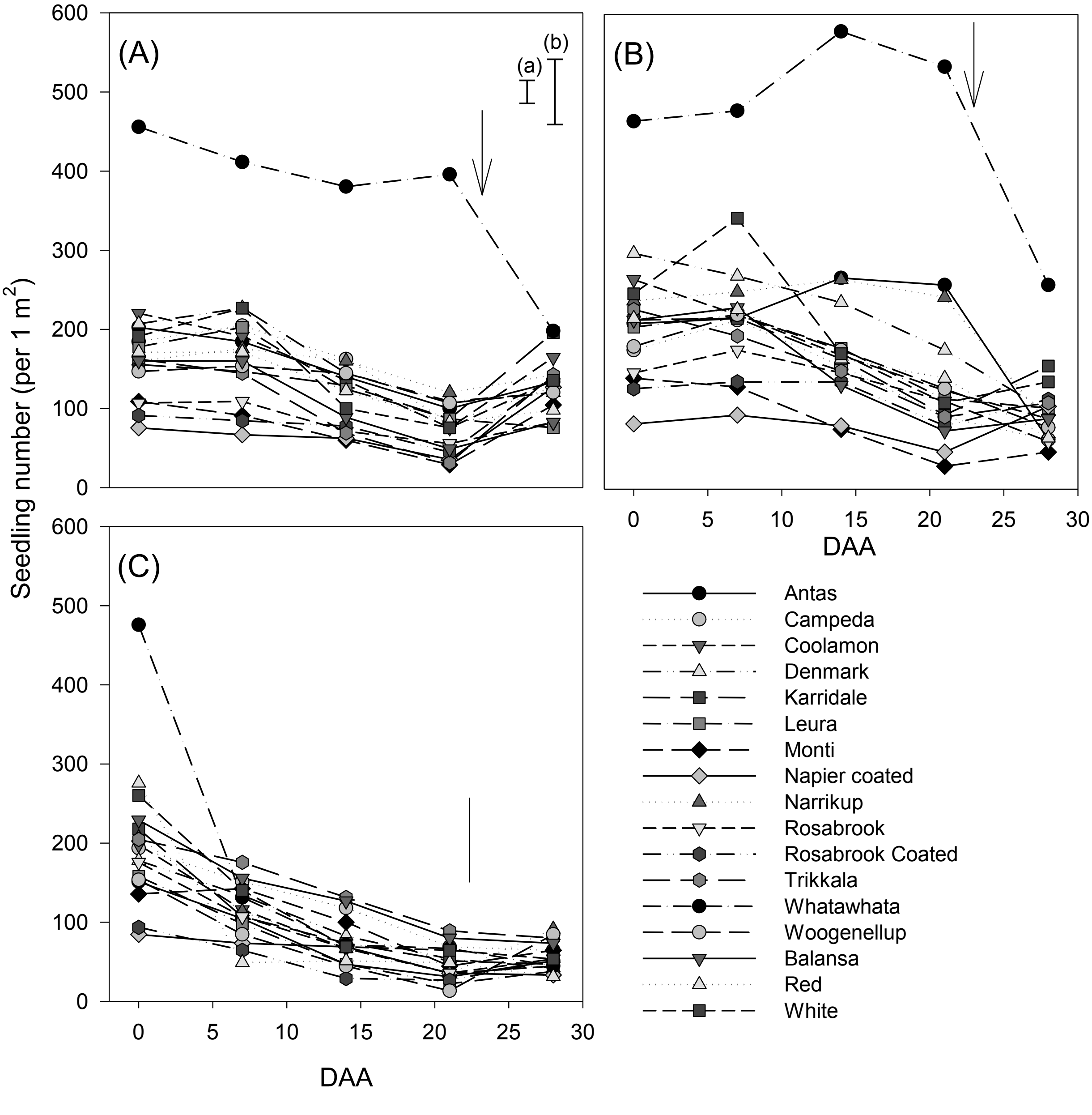

Figure 3.3 Clover seedling number per 1 m^2 for 14 subterranean clover cultivars, balansa, red, and white clovers from 6 April 2016 to 28 days after herbicide application (DAA) at Iversen 1, Lincoln University, Canterbury, New Zealand. (A) is unsprayed unweeded control; (B) is imazethapyr treatment; (C) is imazethapyr + saflufenacil treatment. Error bars are the LSD for (a) time*herbicide interaction; (b) cultivar*herbicide interaction at a fixed time point. Arrows indicate application of 20 mm irrigation.

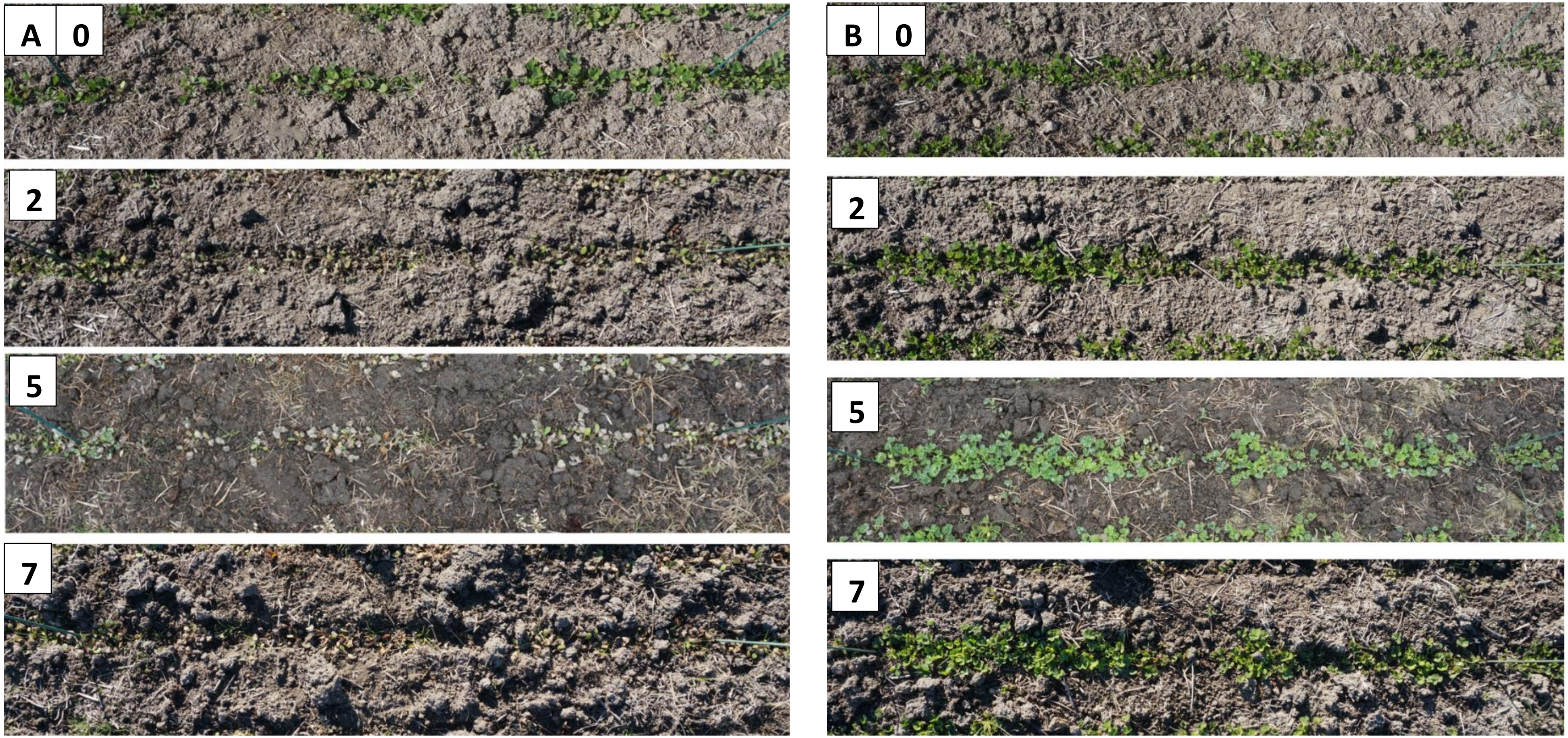

Plate 3.1 (A) 1 m drill row sections of 'Antas' subterranean clover, showing progression of the response to imazethapyr + saflufenacil treatment at 0, 2, 5, and 7 days after application (DAA) on 7 April 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand. (B) 1 m drill row sections of 'Narrikup' subterranean clover showing progression of the response to imazethapyr treatment at 0, 2, 5, and 7 days after application (DAA) on 7 April 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand.

3.3.2 Phytotoxicity assessment

Imazethapyr + saflufenacil had a 100% phytotoxicity effect on all cultivars, severely damaging all cultivars except red clover within the first seven days (Plate 3.1 A). Red clover died by 153 DAA, but still showed a non-commercially viable EWRS score of >7.0 by 60 DAA. EWRS scores were not different for cultivars treated with imazethapyr + saflufenacil (Figure 3.4 B). By 153 DAA, at the time of the first harvest, all plants in imazethapyr + saflufenacil treated plots were dead and the ground was bare (Plate 3.2).

The EWRS score of cultivars treated solely with imazethapyr did show a difference in response. EWRS scores for 'Monti', 'Rosabrook', 'Trikkala', 'Balansa', 'Rosabrook' coated, 'Coolamon', and 'Antas' were 7.0-8.3, and all lower ($P<0.001$) than their controls. These were commercially non-viable scores which indicates a severe phytotoxicity response (Figure 3.4 A, and Plate 3.3, and Plate 3.4).

Imazethapyr treated white, red, and 'Denmark' subterranean clover scored 4.3-5.0, just within the commercially acceptable range for phytotoxicity damage. 'Narrikup', 'Woogenellup', and 'Whatawhata' had final EWRS scores of 1.0-2.0 which were not different to their unsprayed unweeded controls, and commercially acceptable (Figure 3.4 A, and Plate 3.1 B, and Plate 3.5).

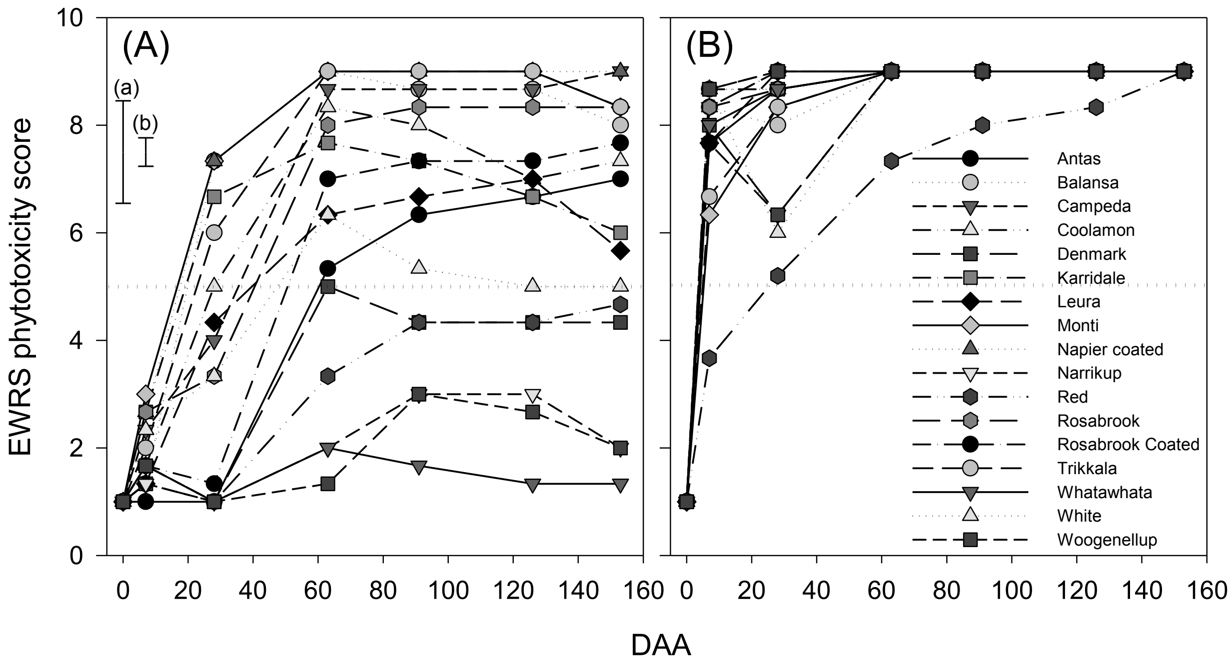

Figure 3.4 EWRS phytotoxicity scores for imazethapyr (A) and imazethapyr + saflufenacil (B) treated clover plots from 0-153 days after application (DAA) at Iversen 1, Lincoln University, Canterbury, New Zealand. Error bars are LSD for (a) cultivar*herbicide interaction change over time; (b) cultivar*herbicide interaction at a fixed time point. Dotted line indicates commercially acceptable EWRS phytotoxicity threshold of 5.0.

Plate 3.2 1 m drill row section of imazethapyr + saflufenacil treated plot at 153 days after herbicide application with a visual EWRS phytotoxicity score of 9. At first harvest on 7 September 2016, at Iversen 1, Lincoln University, Canterbury, New Zealand.

Plate 3.3 Untreated unweeded control 'Antas' plants on 31 August 2016, from Iversen 1, Lincoln University, Canterbury, New Zealand.

Plate 3.4 Spinnaker treated 'Antas' plants on 31 August 2016, with herbicide applied on the 7 April 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand.

Plate 3.5 Spinnaker treated 'Narrikup' plot on 25 October 2016, with herbicide applied on the 7 April 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand.

3.3.3 Plant pubescence and EWRS scores

A correlation of plant pubescence for subterranean clover cultivars and red clvoer with visible herbicide tolerance (EWRS scores) (P<0.001) was identified (Figure 3.5). White and balansa clover were excluded due to their lack of pubescence. 'Narrikup' and 'Whatawhata', with an average leaf and petiole pubescence rating of 2.75, or 'very hairy' had the lowest visible phytotoxicity damage (Plate 3.5), along with 'Woogenellup', which was slightly less hairy with a pubescence score of 2.0, but not different with an EWRS score of 2.0. Glabrous plants, with pubescence scores of 0-1.0 tended towards higher EWRS scores, all greater than the commercially acceptable threshold of 5.0, except 'Denmark', which had an EWRS score of 4.3 (Figure 3.5).

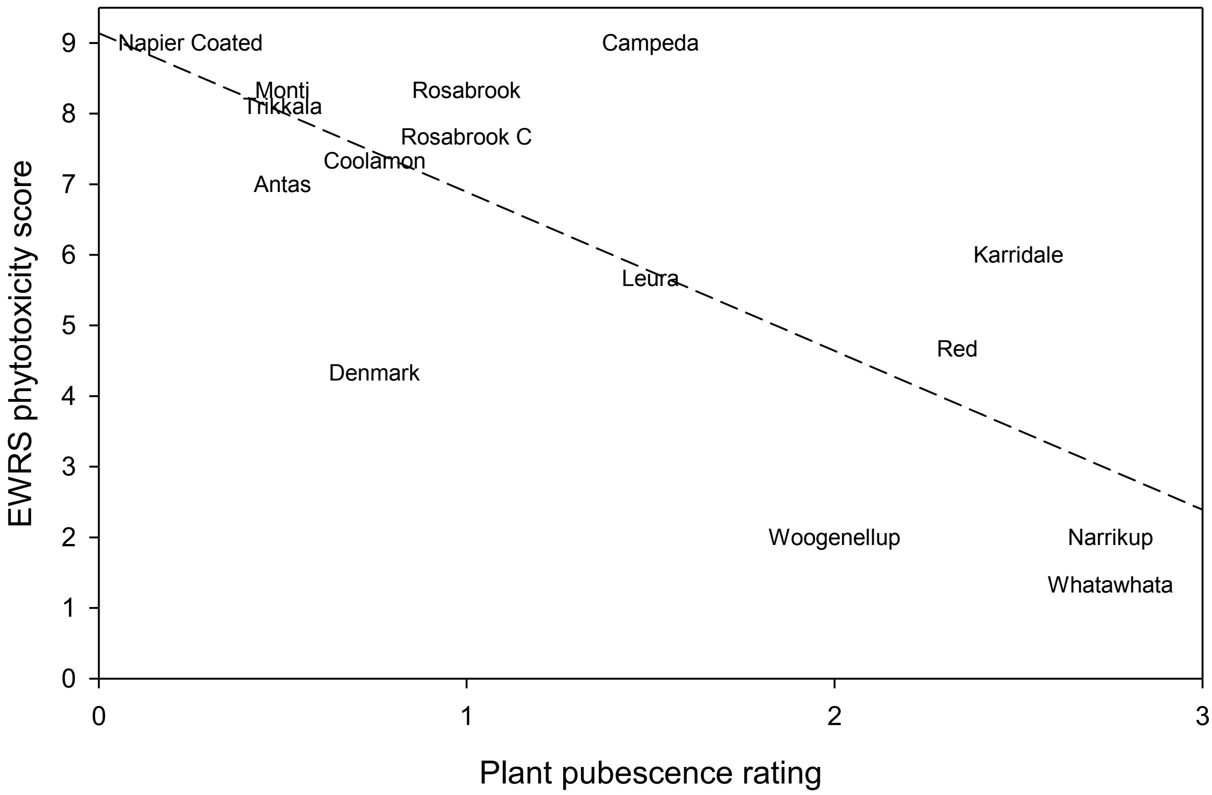

Figure 3.5 EWRS scores for imazethapyr treated clover cultivars plotted against relative plant pubescence scores. Correlation coefficient between EWRS score and plant pubescence = -0.830 (P<0.001).

3.3.4 Dry matter yields

3.3.4.1 Harvest 1: 7 September 2016

Total dry matter yields for harvest 1 were reduced (P<0.001) in the imazethapyr treated plots to be 700 kg DM/ha compared to unsprayed unweeded controls with 3300 kg DM/ha.

For sown clover, there was a herbicide cultivar interaction (P=0.002). Imazethapyr treated 'Woogenellup' was the highest yielding overall, with 2250 kg DM/ha in imazethapyr treated plots, which was greater than the 1340 kg DM/ha in unsprayed unweeded controls (Figure 3.6).

‘Whatawhata’, and ‘Narrikup’ were the only other cultivars to produce higher sown clover yields than their respective controls. Imazethapyr treated ‘Whatawhata’ produced 1830 kg DM/ha, higher (P=0.003) than its unsprayed, unweeded control which produced 610 kg DM/ha. ‘Narrikup’ treated with imazethapyr produced 1710 kg DM/ha compared with 550 kg DM/ha in its unsprayed unweeded control. ‘Antas’, ‘Coolamon’, ‘Karridale’, ‘Leura’, ‘Rosabrook’ coated, red, and white clover produced yields of 60-860 kg DM/ha and were not different between the unsprayed unweeded controls and imazethapyr treatment (Figure 3.6). Sown clover yields <50 kg DM/ha were produced in ‘Trikkala’, balansa clover, ‘Campeda’, ‘Monti’, ‘Napier’ coated, and ‘Rosabrook’ unsprayed unweeded controls and imazethapyr treated plots (Figure 3.6).

Broadleaf weed yields of imazethapyr treatments were <75 kg DM/ha, which were less (P<0.001) than the unsprayed unweeded control at 1500 kg DM/ha.

Annual grass weed yields in imazethapyr treated plots were 140 kg DM/ha, which was less (P<0.001) than their respective controls, which produced 1370 kg DM/ha (Figure 3.6). Dead matter yields were reduced (P<0.001) in imazethapyr treated plots at <50 kg DM/ha compared with unsprayed unweeded controls with 130 kg DM/ha.

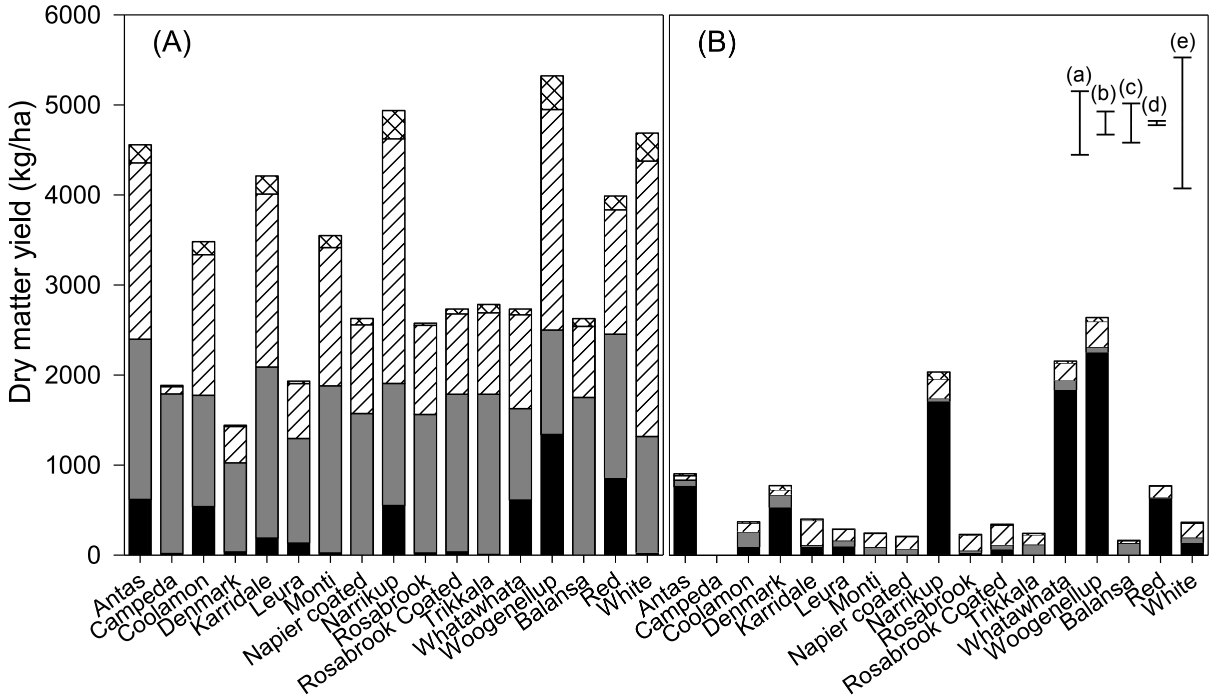

Figure 3.6 Mean dry matter yields and composition for growth of 17 clover cultivars from 1 March 2016 to 9 September 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand for sown clover (■), broadleaf weed (■), grass weed (▨) and dead matter (⊠) components of cultivar plots. (A) is unsprayed unweeded control; (B) is imazethapyr treated. Error bars are the LSD for (a) herbicide*cultivar interaction for sown clover yields; (b) main effect of herbicide on broadleaf weed yields; (c) main effect of herbicide on grass weed yields; (d) main effect of herbicide on dead matter; (e) main effect of herbicide on total dry matter yields.

3.3.4.2 Harvest 2: 25 October 2016

Due to death, non-tolerant cultivars, 'Trikkala', balansa clover, 'Campeda', 'Monti', 'Napier' coated, and 'Rosabrook' were removed from evaluation after the first harvest and no longer harvested. There was no cultivar*herbicide interaction for the remaining cultivars (P=0.254). Sown clover yields of imazethapyr treatments were higher (P<0.001) at 1080 kg DM/ha than controls with 320 kg DM/ha in the second harvest after hard grazing. Imazethapyr treated 'Whatawhata', 'Woogenellup', 'Narrikup', and 'Denmark' had the highest (P<0.001) sown clover yields, of 1240-2730 kg DM/ha. 'Coolamon', 'Rosabrook' coated, 'Leura', Karridale, 'Antas' and white clover were the lowest (P<0.001) yielding less than 900 kg DM/ha of sown clover (Figure 3.7).

Broadleaf weed yields were less than 200 kg DM/ha in imazethapyr treated plots, which was reduced (P<0.001) compared with 800 kg DM/ha in their controls (Figure 3.7). Grass weed yields averaged 1300 kg DM/ha in unsprayed unweeded control plots, which was reduced (P<0.001) to 500 kg DM/ha by Spinnaker (Figure 3.7). Dead matter yields were not different (P=0.094) between unsprayed unweeded controls and imazethapyr treatments at <50 kg DM/ha. Total dry matter yields were reduced (P<0.001) in imazethapyr treatments at 1800 kg DM/ha, compared to unsprayed unweeded controls with 2600 kg DM/ha (Figure 3.6).

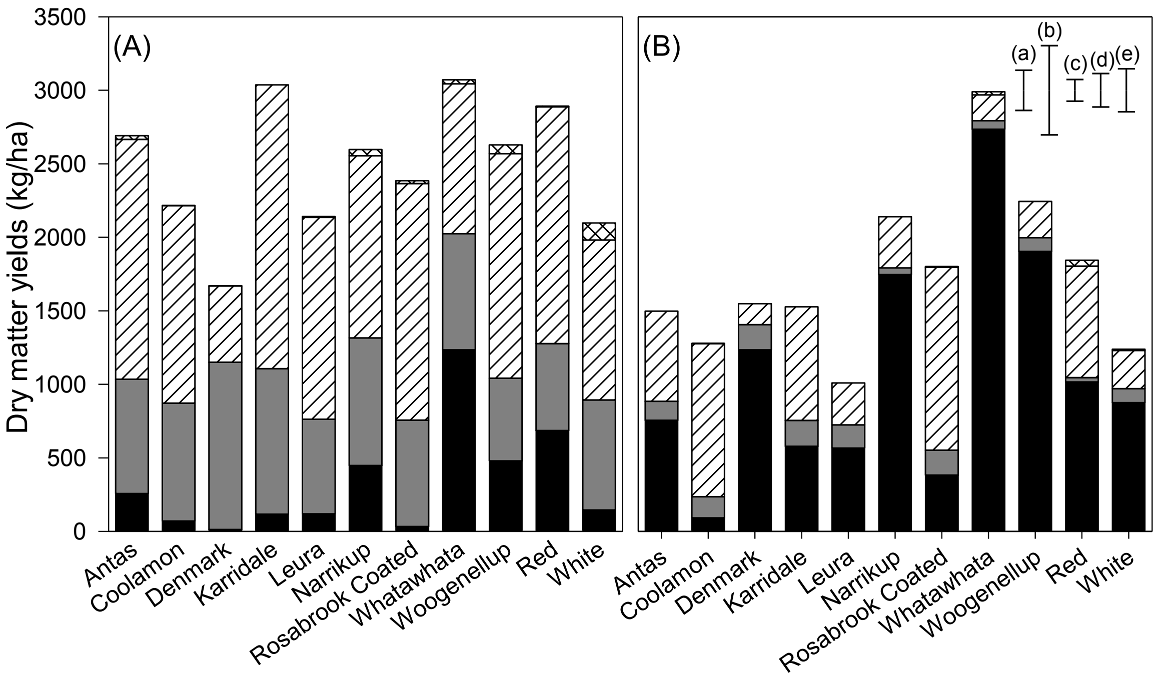

Figure 3.7 Mean dry matter yields and composition for growth of 11 clover cultivars from 19 September to 25 October 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand for sown clover (■), broadleaf weed (■), grass weed (▨) and dead matter (⊠) components of cultivar plots. (A) is unsprayed unweeded control; (B) is imazethapyr treated. Error bars are the LSD for (a) main effect of herbicide on sown clover yields; (b) main effect of cultivar on sown clover yields; (c) main effect of herbicide on broadleaf weed yields; (d) main effect of herbicide on grass weed yields; (e) main effect of herbicide on total dry matter yields.

3.3.4.3 Total season yields

Total dry matter yields were reduced (P<0.001) in imazethapyr treatments, at 2700 kg DM/ha, compared with 6100 kg DM/ha in unsprayed unweeded contols (Figure 3.8). There was a cultivar*herbicide interaction for sown clover dry matter yields (P=0.012). The highest sown clover total dry matter yields were seen in 'Whatawhata', 'Woogenellup', and 'Narrikup' treated with imazethapyr. Their yields of 3500 – 4500 kg DM/ha by 25 October 2016 were higher (P<0.001) than all other cultivars. All imazethapyr treatments which survived, outperformed their respective controls to some degree, except 'Coolamon'. Only 'Whatawhata', 'Woogenellup', 'Narrikup' and 'Denmark' produced higher (P<0.001) sown clover yields compared to their controls. 'Whatawhata' control, 'Woogenellup' control, 'Denmark' treated with imazethapyr, Red imazethapyr, Red control, and 'Antas' treated with imazethapyr, ranged from 1520-1850 kg DM/ha (Figure 3.8).

Broadleaf weed yields were <200 kg DM/ha in imazethapyr treated plots, lower (P<0.001) than 2200 kg DM/ha in the unsprayed unweeded controls. Grass weed (annual ryegrass, phalaris, and annual brome) yields averaged 3000 kg DM/ha in the control plots but this was reduced (P<0.001) to 650 kg DM/ha by imazethapyr. The grass weeds remaining in imazethapyr treated plots were predominantly ryegrass. Dead matter was <50 kg/ha in imazethapyr treated plots, less (P<0.001) than the unsprayed unweeded controls with 200 kg/ha.

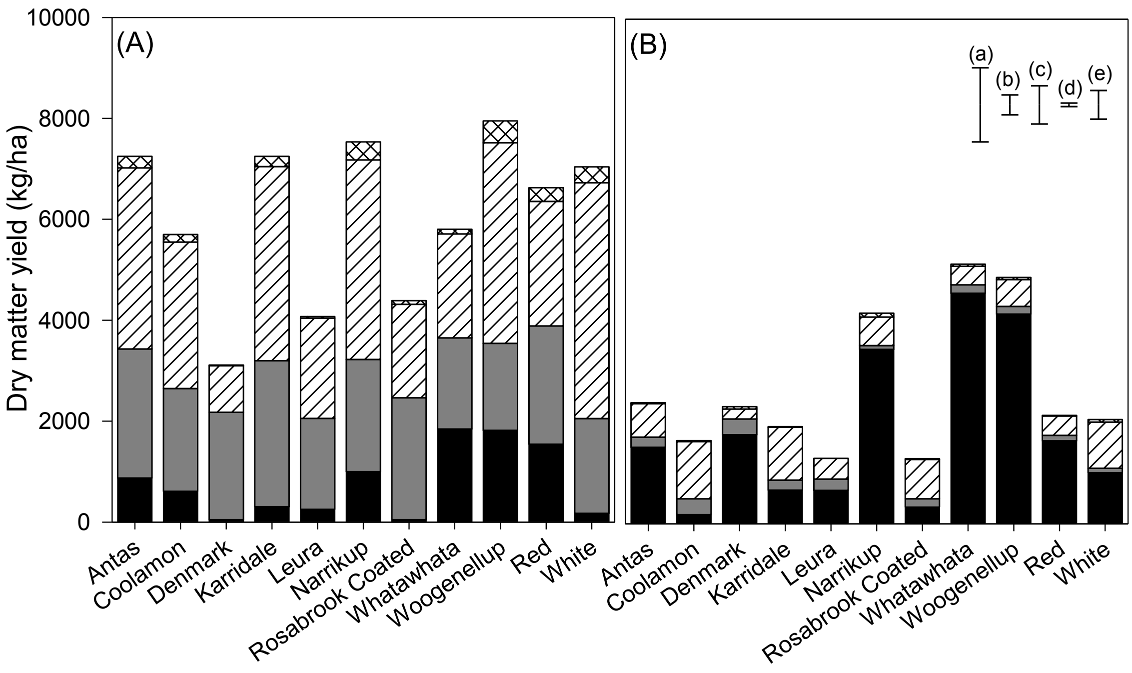

Figure 3.8 Total mean dry matter yields and composition for growth of 11 clover cultivars from 1 March to 25 October 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand for sown clover (■), broadleaf weed (■), grass weed (▨) and dead matter (▩) components of cultivar plots. (A) is unsprayed, unweeded controls; and (B) is imazethapyr treated cultivar plots. Error bars are LSD for (a) herbicide*cultivar interaction in sown clover; (b) main effect of herbicide on broadleaf weed yields; (c) main effect of herbicide on grass weeds yields; (d) main effect of herbicide on dead matter; (e) main effect of herbicide on total dry matter yields.

3.3.5 EWRS scores and yields

A correlation of EWRS scores and harvest yields identified a negative association of yields with EWRS scores ($P<0.001$); as EWRS scores increased, sown clover dry matter yields decreased (Figure 3.9). 'Whatawhata', 'Woogenellup', and 'Narrikup' all had low EWRS scores 1.0-2.0 and these correlated with high yields of 3500-4500 kg DM/ha. 'Denmark' and red clover had average EWRS scores at 4.0-5.0, and at 1500-1800 kg DM/ha, lower yields than 'Whatawhata', 'Woogenellup' and 'Narrikup'. 'Antas' had a higher EWRS score at 7.0, but a higher yield than expected, at ~1500 kg DM/ha. All remaining cultivars had high EWRS scores, above the commercial threshold, which correlated well with their <1000 kg DM/ha total season yields.

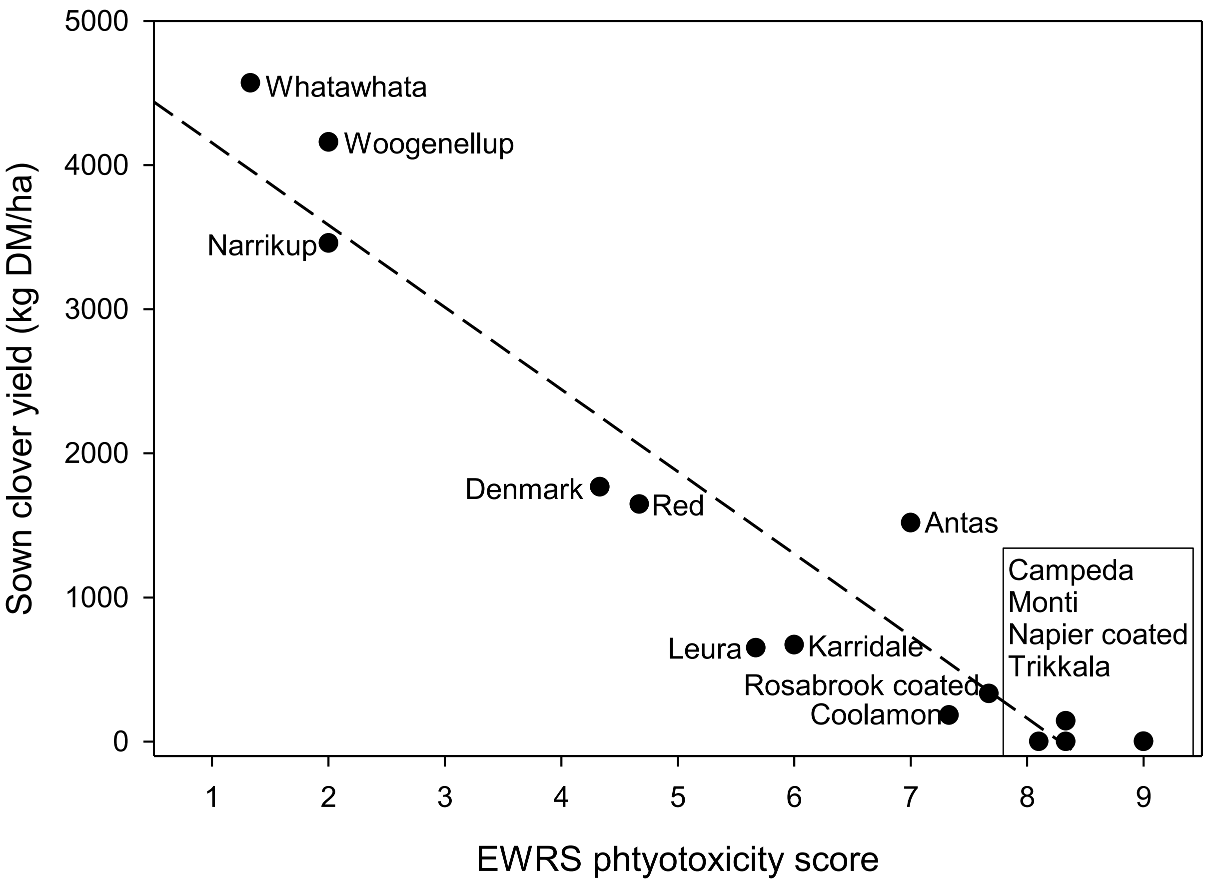

Figure 3.9 Imazethapyr treated clover cultivar season dry matter yields plotted against EWRS phytotoxicity scores. Correlation coefficient between sown clover yield and EWRS phytotoxicity score = -0.901 ($P<0.001$).

3.4 Discussion

3.4.1 Seedling establishment

Seedling establishment was highest for 'Whatawhata' (Figure 3.2), a New Zealand selected line. The high seedling establishment of the 'Whatawhata' line was due to the five-fold increase in sowing rate, at 100 kg/ha. The 'Whatawhata' line was sown at such a high rate due to the seed provided containing 30% broken seeds, and in *in vitro* germination tests which used only undamaged seeds, only 78% germinated (Table 3.1). As a result, it was assumed that field performance would be impacted, with less than 50% germination. However, this field response was therefore a surprise and confounds discussion of this cultivar.

The seedling emergence (Figure 3.2) prior to application suggests that most of the sown seed emerged (Table 3.1), and establishment was a success. Any further effects are a result of herbicide applications and environmental impact on persistence.

3.4.2 Imazethapyr + saflufenacil

Based on the results of this experiment, imazethapyr + saflufenacil should not be used as a post-emergence herbicide on subterranean clover. All clover species and cultivars showed a rapid phytotoxicity response to the imazethapyr + saflufenacil application with seedling numbers reduced by 70% at 28 days after application (Figure 3.3 C). Red clover showed slightly more tolerance than all other sown legumes, but even it had still senesced completely by the first harvest in September. The product also showed a prolonged residual, as any clover seed and weeds that germinated during the experimental period died rapidly after emergence, with ground still bare in November. The rapid response to treatment application which was observed in the imazethapyr + saflufenacil treatments is probably due to the PPO inhibiting mechanism of action of the saflufenacil. PPO inhibition essentially prevents a plant from protecting itself from light. This was visible in the imazethapyr + saflufenacil treated plants within two days of application, where all vegetative areas of the plant had bleached and were becoming necrotic, with only cotyledons remaining, but showed signs of chlorosis and necrosis. After five days, post light rain, plants had advanced to look slightly more necrotic, but green tissue appeared somewhat more vigorous. By seven days after herbicide application, most cotyledons had necrotised, with little visible green tissue remaining (Plate 3.1).

3.4.3 Phytotoxicity effects and sown clover yields

Subterranean clover cultivars exhibited a strong cultivar*herbicide response to imazethapyr treatment. Subterranean clover cultivars 'Campeda', 'Monti', 'Napier' coated, 'Rosabrook' and 'Trikkala', and 'bolta' balansa clover showed no tolerance of imazethapyr treatment, with high commercially unacceptable EWRS scores (Figure 3.4). They had low correlating first harvest yields, of

<50 kg/ha sown clover in imazethapyr treated plots. Of these, subterranean clover cultivars 'Campeda', 'Trikkala', and 'Bolta' balansa clover had no difference in seedling establishment per m^2 from all other cultivars which did yield higher than 50 kg/ha, except 'Whatawhata' (Figure 3.3). However, 'Monti', 'Napier' coated, and 'Rosabrook' had lower seedling establishment than all cultivars except 'Woogenellup' and 'Rosabrook' coated. This suggests they may have had compromised establishment due to the lower than average autumn rainfall (Figure 3.1). As a consequence these seedlings may have been under more stress at the time of application, increasing their susceptibility to the imazethapyr treatment. The coating on 'Rosabrook' coated may have caused it to emerge at a slower rate than 'Rosabrook', allowing it to emerge into slightly more favourable conditions.

Seedling responses of those treated with imazethapyr were not different to each other, or the unsprayed unweeded controls, with 40% seedling loss per m^2 by 28 DAA (Figure 3.3). 'Whatawhata' had the greatest reduction in seedling number, with 50% lost by 28 DAA, but this was observed in both the unsprayed unweeded control, and the imazethapyr treatment. This occurrence in both surviving treatments is most likely a self-thinning mechanism, where plants which are overcrowded cannot raise their leaves above the canopy to access light, and die (Hamilton et al. 1995). Imazethapyr treated plants began to show visible symptoms at ~28 DAA, but by this point plants had about 10 trifoliate leaves, and were too large to allow accurate seedling counts. Any further damage was noted using the EWRS phytotoxicity score system.

EWRS scores provided an indication of relative cultivar tolerance of imazethapyr (Figure 3.9). 'Woogenellup', 'Whatawhata', 'Narrikup' and 'Denmark' subterranean clovers, as well as red, and white clover all had EWRS scores below the commercially unacceptable threshold of 5.0 (Table 2.1), and yielded the same as, or higher than their unsprayed unweeded controls. 'Narrikup', 'Wooogenellup', and 'Whatawhata', with EWRS scores not different to the unsprayed unweeded controls, were the only cultivars to produce total season sown clover yields higher than their unsprayed unweeded controls. 'Denmark' and white clover recovered after the first harvest, but total season sown clover yields were not different to their unsprayed unweeded controls. The only cultivar to have a somewhat confounded EWRS score was 'Antas', with an EWRS score of 7.0 (Figure 3.4, and Plate 3.4) and first harvest sown clover dry matter yields of 860 kg/ha, which were not different to the unsprayed unweeded control (Figure 3.6, and Plate 3.3). With such a high EWRS score, it was expected to see a significant reduction in yield, however, despite the visible herbicide damage there was no effect on yields compared to unsprayed unweeded controls. This lack of change in yield is due to 'Antas' usually being a very productive cultivar (Lucas *et al.* 2015). However it was likely affected by the month of no rainfall following herbicide application (Figure 3.1), and under too much stress to grow at its usual high rate. The inability of 'Antas' to occupy the space provided means weed control was not an advantage, as there was no adjustment in total dry matter yields, and any palatable weeds within the unsprayed unweeded controls are still capable of providing feed.

Imazethapyr treated ‘Karridale’, ‘Leura’, ‘Rosabrook’ coated, and ‘Coolamon’ lines all produced >50 kg DM/ha at their first harvest. These low yields were no different to their unsprayed unweeded controls. However they were assigned potentially tolerant, with a chance of recovery and were harvested again in the second harvest to identify any recovery that occurred post-grazing. All four cultivars recovered from grazing to yield higher in the second harvest than in the first, with 100-600 kg DM/ha. Clover yields of these four cultivars over the entire season showed no difference between unsprayed unweeded control and imazethapyr treated plots. These cultivars which showed later season tolerance to imazethapyr treatment may be more tolerant when treated in less water limited conditions, or treated at a later growth stage, such as 6+ trifoliate leaves. However a later treatment date allows longer for weeds to establish themselves, and by the time weeds have been eliminated, conditions which allow for germination may have passed, especially in autumn sown crops like subterranean clover. Evers *et al.* (1993) has previously noted that subterranean clover yield reductions as a result of herbicide application can differ widely depending on the growing season conditions, with environmental factors such as soil moisture, soil organic matter, soil clay content, soil pH, rainfall, temperature, frost and relative humidity all having the potential to influence a plant response to herbicide treatment. That is, any condition which stresses the crop, such as extremes in temperature or moisture can reduce the tolerance of the crop to herbicide treatment (Dear *et al.* 1995; Taiz & Zeiger 2010). This has been observed before in subterranean clovers (Section 2.6).

With an early March sowing date, and germination in late March, a high yield was expected based on previous research (Moot *et al.* 2003). However delayed autumn rainfall (Figure 3.1), for three weeks after herbicides were applied, meant seedlings were treated in dry stressed conditions, and had no reprieve until irrigation was applied in May (Figure 3.3), when seedling numbers for all treatments except ‘Whatawhata’ and ‘Leura’ increased in the unweeded unsprayed control. In imazethapyr treated plots, the overall trend for plant numbers was to remain steady, however the poorer established subterranean clovers ‘Karridale’, ‘Monti’, ‘Napier’ coated, ‘Rosabrook’ coated and ‘Trikkala’, and balansa and white clover all showed increases in seedling number following irrigation, suggesting they were severely impacted by the dual stresses, and some seed did not germinate in the original sowing.

3.4.4 Broadleaf weed control

Imazethapyr treatment achieved >90% weed control over the whole season, and controlled all weeds within the experiment (Table 3.4) except mallow, and dock. However, growth of these was greatly reduced compared to the unsprayed unweeded controls (Figure 3.8).

Imazethapyr is an imidazolinone herbicide, with an acetolactate synthase inhibiting mode of action (Section 2.5.4). ALS herbicides are broad spectrum selective herbicides, used at low rates, and readily absorbed by both foliage and roots for translocation via phloem and xylem to their site of action, plant growth points. To be effective, plants must be in active growth stages, and so early seedling application offers the best weed control, but can also increase sown plant loss if they are susceptible. Susceptibility at the seedling stage can cause fatal losses, as shown by the clover yields of 'Campeda', 'Trikkala', Balansa, 'Monti', 'Napier' coated, and 'Rosabrook'. If plants were treated with imazethapyr at a later growth stage, such as the 6-10 trifoliate leaf stage recommended by Evans *et al.* (1989), losses may not have been so extreme. The result of 'Trikkala' is in direct contrast to all previous herbicide tolerances found (Dear *et al.* 1992; Dear & Sandral 1999; Dear *et al.* 1995; Sandral & Dear 2005).

3.4.5 Plant pubescence

'Woogenellup', 'Narrikup' and 'Whatawhata' benefitted from imazethapyr treatment, with sown clover yields 1500-2500 kg DM/ha higher than their unsprayed unweeded controls (Figure 3.8). A physiological trait which may explain this herbicide tolerance is relative plant pubescence (Cobb & Reade 2010), as 'Narrikup', 'Whatawhata', and 'Woogenellup' have higher plant hairiness ratings than cultivars such as 'Monti', 'Denmark', and 'Trikkala'. Hairiness was assessed as a covariate of yield and EWRS score for all cultivars, and a negative association between plant pubescence and EWRS scores was found, where EWRS scores were higher in glabrous than highly pubescent plants (Figure 3.5). Those cultivars which were hairy had a greater advantage over glabrous plants, as they had an extra protective layer which the chemical had to penetrate before the plant absorbed the herbicide, potentially reducing the amount which was taken up by the plant (Streibig 2003). As the imazethapyr was tank mixed with Hasten™, which is an oil surfactant in the wetter spreader class, this result cannot be attributed to a lack of adjuvant reducing uptake potential.

3.4.6 Total dry matter yields

Based on the result of this experiment, imazethapyr substantially reduced total dry matter yields. The weed control it provided was effective, but only advantageous for tolerant cultivars capable of exploiting the extra space available free from weed competition.

3.5 Conclusions

Saflufenacil mixed with imazethapyr successfully controlled broadleaf weed for over four months, but was not a suitable post-emergence herbicide for sub-clover containing pastures.

EWRS scores can be used to quantify visual phytotoxicity effects of imazethapyr treatment in subterranean clover stands.

There was a cultivar*herbicide response of subterranean clover cultivars to Spinnaker treatment, and this appears to have an association with plant pubescence. 'Woogenellup' and 'Narrikup', showed high tolerance to imazethapyr treatment. ‘Whatawhata’, Denmark’, and ‘Antas’ subterranean clovers showed tolerance to imazethapyr treatment.

Overall, total dry matter yields of subterranean clover and weeds were greatest in unsprayed unweeded controls, so if weeds are palatable, spraying is not required. If herbicide tolerance of a cultivar is uncertain, then not spraying with imazethapyr is recommended, and weed yields of 2000 kg DM/ha are better than no dry matter yields. However, lower sown clover yields in weedy areas are likely to affect subsequent seed set and regeneration, which were not a part of this study.

4 EXPERIMENT 2: EFFECT OF IMAZETHAPYR AND SAFLUFENACIL ON CLOVER CONTAINING PASTURE MIXTURE ESTABLISHMENT AND PRODUCTIVITY

4.1 Introduction

In New Zealand, subterranean clover is often sown as a companion legume with grasses in mixed swards. The aim is to increase sward quality directly and indirectly through increased nitrogen fixation (Lucas *et al.* 2015). The 'MaxClover' grazing experiment at Lincoln University from February 2002 until 2011 identified cocksfoot-subterranean clover mixtures as the most successful mixed sward in a dryland environment. Yields of cocksfoot subterranean clover pastures were second only to lucerne monocultures for the experimental period. The clover-cocksfoot pasture mixtures produced 8700-13000 kg DM/ha annually, with a subterranean clover component of 2400-3700 kg DM/ha in six of the nine experimental years (Mills *et al.* 2014). A field trial at Lincoln University in 2014 investigated cocksfoot with 10 subterranean clover cultivars and found that 'Antas' and 'Narrikup' produced the highest total early spring DM yields in September, with 750 kg DM/ha ((Lucas *et al.* 2015)). In November, 'Antas' was again one of the highest yielding cultivars, along with 'Woogenellup' and 'Leura', producing yields of 2700 kg DM/ha. 'Narrikup' was one of the lowest yielding cultivars in this later harvest, along with 'Denmark', 'Rosabrook' and 'Monti', with 1500 kg DM/ha (Lucas *et al.* 2015). To maximise yields at establishment and through the spring, weed control is important.

This experiment will evaluate the effect of two recommended (BASF, G Hagerty, pers comm., 27 January 2016). post-emergence herbicides on five clover-cocksfoot mixtures in the establishment phase. This will achieve Ojective 2 (Figure 1.1), and identify the seedling stage tolerance of the mixed pastures to imazethapyr, an ALS inhibiting herbicide (Section 2.5.4), and imazethapyr + saflufenacil, a PPO inhibiting herbicide (Section 2.5.5).

4.2 Materials and methods

Materials and methods were described in Chapter 3, Section 3.2.

4.2.1 Experimental design

Three subterranean clover cultivars: 'Antas', 'Denmark', 'Narrikup' at rates of 20 kg/ha; plus 'Nomad' white clover and 'Bolta' balansa clover at rates of 10 kg/ha, were drilled into cultivated soil with 'Greenly II' cocksfoot at 2 kg/ha (Table 3.1, Table 3.5). Plots were 8 x 2.1 m, with five replicates in a randomized complete block design. At the 3-4 trifoliate leaf stage, the two herbicide treatments, (Table 3.6) were applied in 2 m strips across plots, randomised for each replicate. This left a 2 m unsprayed, unweeded control strip adjacent to each sprayed plot.

4.3 Results

4.3.1 Seedling establishment

Seedling numbers prior to herbicide application were 200-220 per m^2 and not different (P=0.080) among cultivars but reduced (P<0.001) over time (Figure 4.1). Imazethapyr + saflufenacil treated seedlings dropped to 40 per m^2, which was less (P<0.001) than the 110 per m^2 for the unsprayed unweeded controls at 28 DAA (Figure 4.1, A, and C). Imazethapyr treated seedlings were not different to the unsprayed unweeded controls at 28 DAA with no difference among cultivars (Figure 4.1 A, and B).

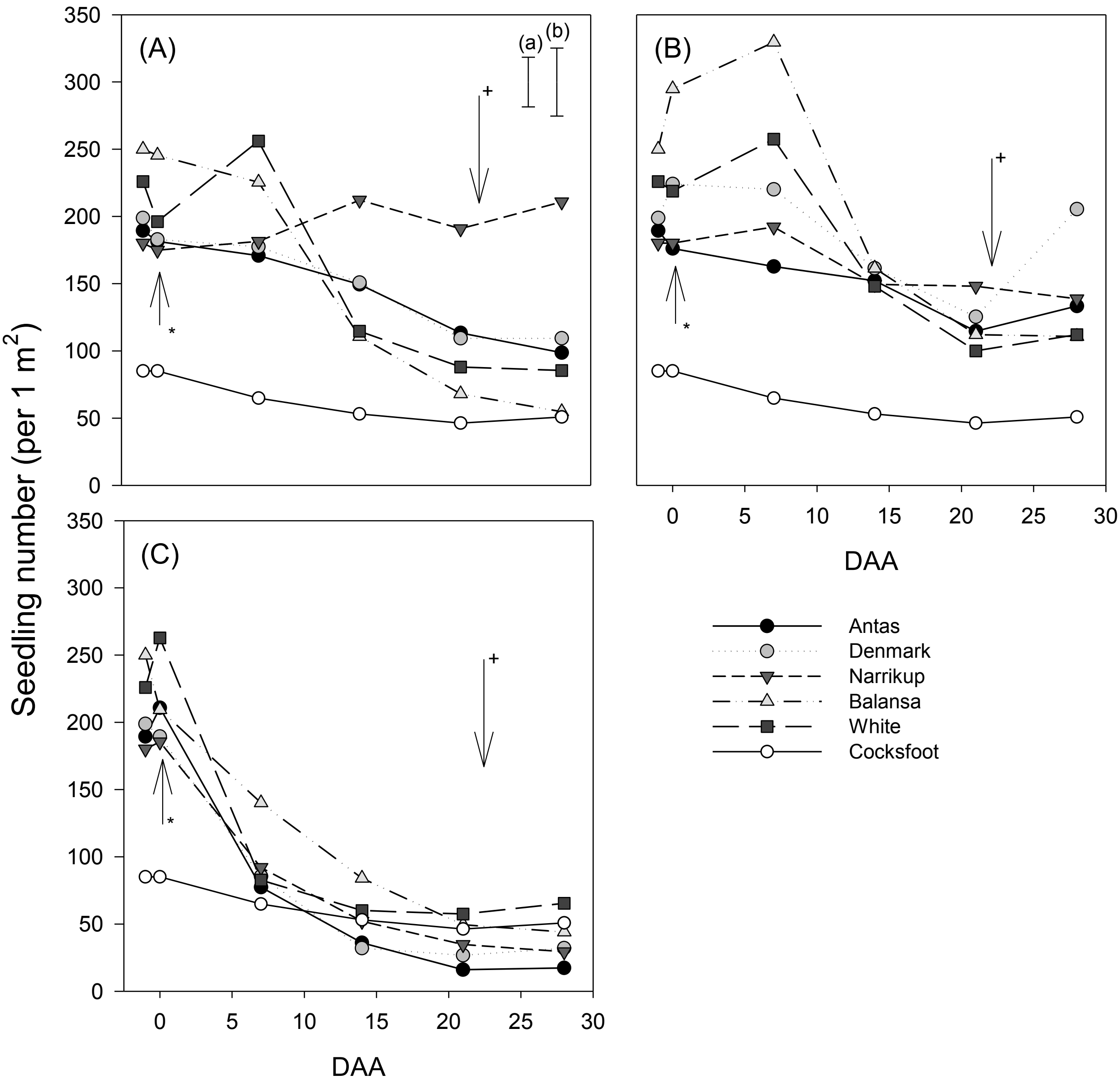

Figure 4.1 Seedling number per m^2 for five cultivars and cocksfoot for 28 days after application (DAA) of (A) unsprayed unweeded control, (B) imazethapyr treatment and (C) imazethapyr + saflufenacil treatment from 6 April 2016, at Iversen 1, Lincoln University, Canterbury, New Zealand. Error bars are LSD for (a) main effect of herbicide on seedling number at a single time point; (b) herbicide*time interaction for seedling number. Arrows with * indicate herbicide application; arrows with + indicates application of 20 mm irrigation.

4.3.2 Phytotoxicity assessment

EWRS scores for all imazethapyr + saflufenacil treated plots were 8-9 within 7 DAA. This recovered to 2.0-4.0 for white clover and 'Narrikup' by 28 DAA, after an irrigation event, however by 63 DAA, all cultivars had an EWRS score of 9.0, meaning the imazethapyr + saflufenacil mix was not safe for subterranean clover (Figure 4.2 B).

Imazethapyr treated balansa clover showed no tolerance, with an EWRS score of 5.0 by 28 DAA, and this increased (P<.001) to a final score of 7.0 by 154 DAA (Figure 4.2 A). All other imazethapyr treated cultivars were not different to controls at 28 DAA, but all EWRS scores increased (P<0.001) by day 63, at which point only 'Narrikup' with a score of 3.0 was within the commercially acceptable bounds. This score did not change for the next 100 days. By 153 DAA, 'Antas' and white clover had recovered (P<0.001) with EWRS scores of 4.0-5.0, within the commercially acceptable threshold (Figure 4.2).

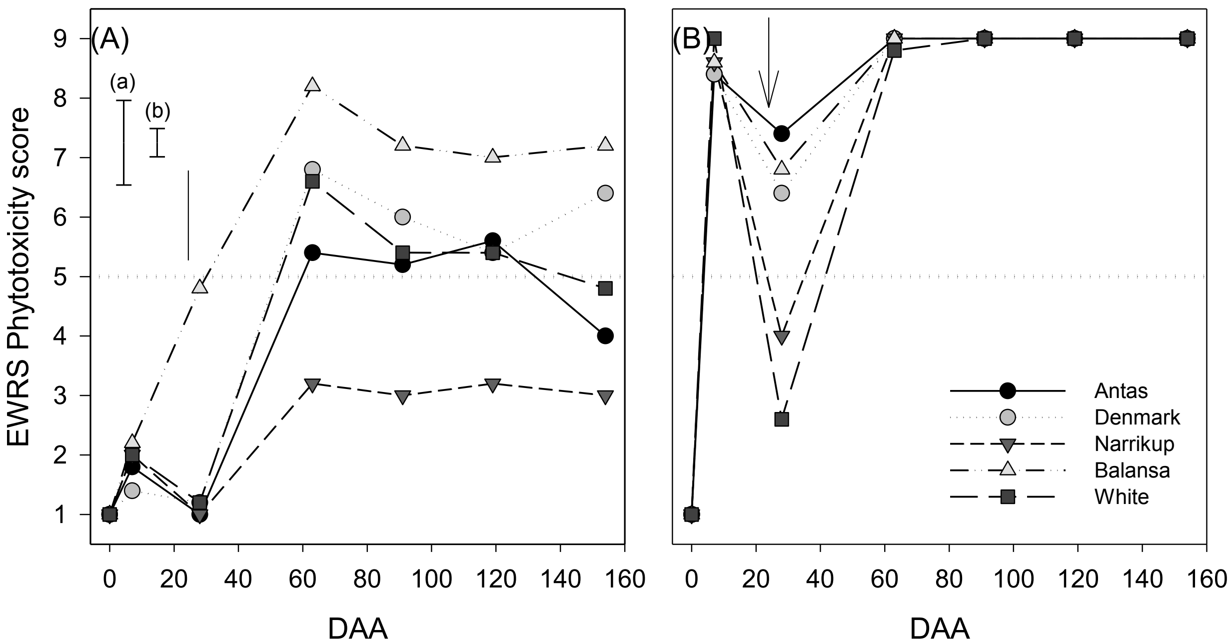

Figure 4.2 EWRS phytotoxicity scores relative to unsprayed unweeded controls for all cultivars in (A) imazethapyr and (B) imazethapyr + saflufenacil treatments for 154 days after treatment application (DAA) at Iversen 1, Lincoln University, Canterbury, New Zealand. Error bars are LSD for (a) herbicide*time interaction; (b) herbicide*cultivar interaction at a single time point. Dotted line indicates commercial EWRS score threshold; arrows indicate application of 20 mm irrigation.

4.3.3 Dry matter yields

4.3.3.1 Harvest 1: 9 September 2016

Sown clover yields were not different (P=0.125) between imazethapyr treated clovers and unsprayed unweeded controls. 'Narrikup' yields of 1100 kg DM/ha were higher (P=0.006) than all other cultivars which produced <250 kg DM/ha (Figure 4.3).

Sown cocksfoot grass yields in imazethapyr treated plots were <350 kg DM/ha, lower (P=0.003) than controls with 1100 kg DM/ha. This is consistent with the results of Experiment 1, where imazethapyr controlled annual grass weeds (Figure 3.8).

Broadleaf weed yields of imazethapyr treatments were <50 kg DM/ha, which was less (P<0.001) than their respective controls with 400 kg DM/ha. Grass weed yields of <100 kg DM/ha were lower (P=0.014) in imazethapyr treated plots than their controls which had grass weed yields of 1100 kg DM/ha. Imazethapyr treatments yielded <50 kg DM/ha dead matter, lower (P=0.005) than the unsprayed unweeded controls, which had 300 kg DM/ha (Figure 4.3).

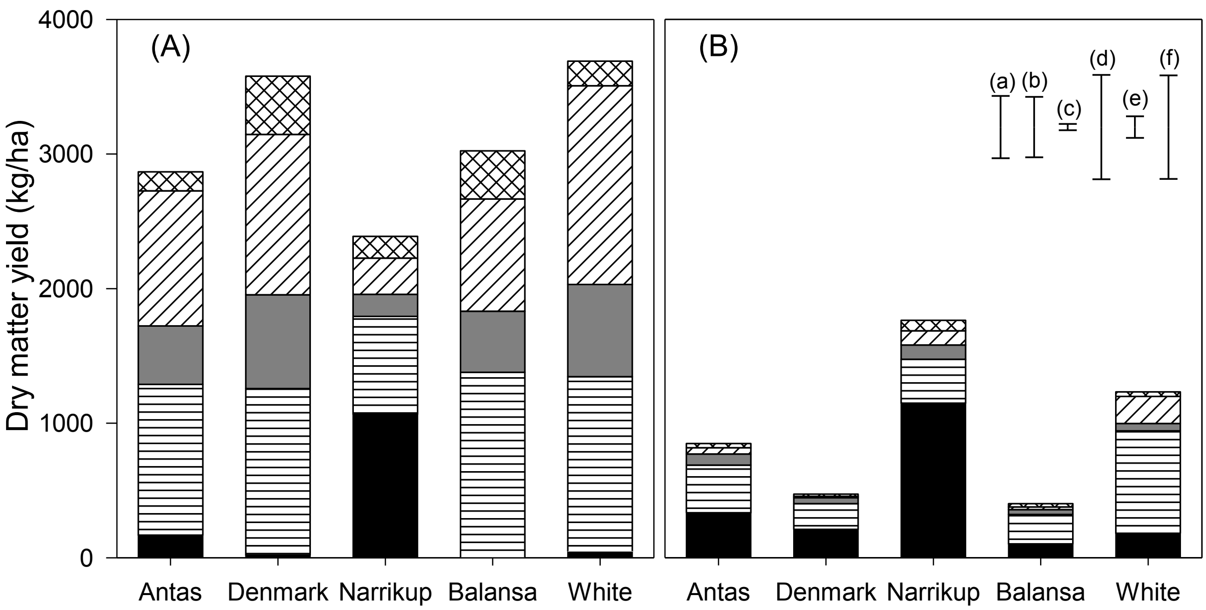

Figure 4.3 Mean dry matter yields and composition for growth of five clover cultivars from 1 March to 9 September 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand for sown clover (■), cocksfoot (▤), broadleaf weed (■), grass weed (▨) and dead matter (▩) components of cultivar plots. (A) is unsprayed, unweeded controls, and (B) is imazethapyr treatments. Error bars are LSD for main effect of (a) cultivar on clover yield; (b) herbicide on cocksfoot; (c) herbicide on broadleaf weeds; (d) herbicide on grass weeds; (e) herbicide on dead matter; (f) herbicide on total dry matter yields.

4.3.3.2 Harvest 2: 27 October 2016

Total mean dry matter yields were >1000 kg DM/ha higher (P<0.001) in unsprayed unweeded controls at 3400 kg DM/ha than imazethapyr treated plots with 2300 kg DM/ha (Plate 4.1). There were no differences among cultivars (P=0.105).

Sown clover yields of imazethapyr treatments were higher (P=0.001) with 750-1800 kg DM/ha, than their respective unsprayed unweeded controls, except in balansa clover, which had no difference in yield between imazethapyr and the unsprayed unweeded control, both producing <100 kg DM/ha (Figure 4.4).

Sown cocksfoot grass yields were 1000-1650 kg DM/ha after grazing and were not different (P=0.099) between imazethapyr plots and their unsprayed unweeded controls.

Broadleaf weed yields were 100-250 kg DM/ha with no difference (P=0.128) between imazethapyr and unsprayed unweeded controls. Grass weed yields were higher (P=0.005) in unsprayed unweeded controls, with 1300 kg DM/ha, while imazethapyr treatments had 250 kg DM/ha (Figure 4.4). Remaining grass weeds were predominantly annual ryegrass.

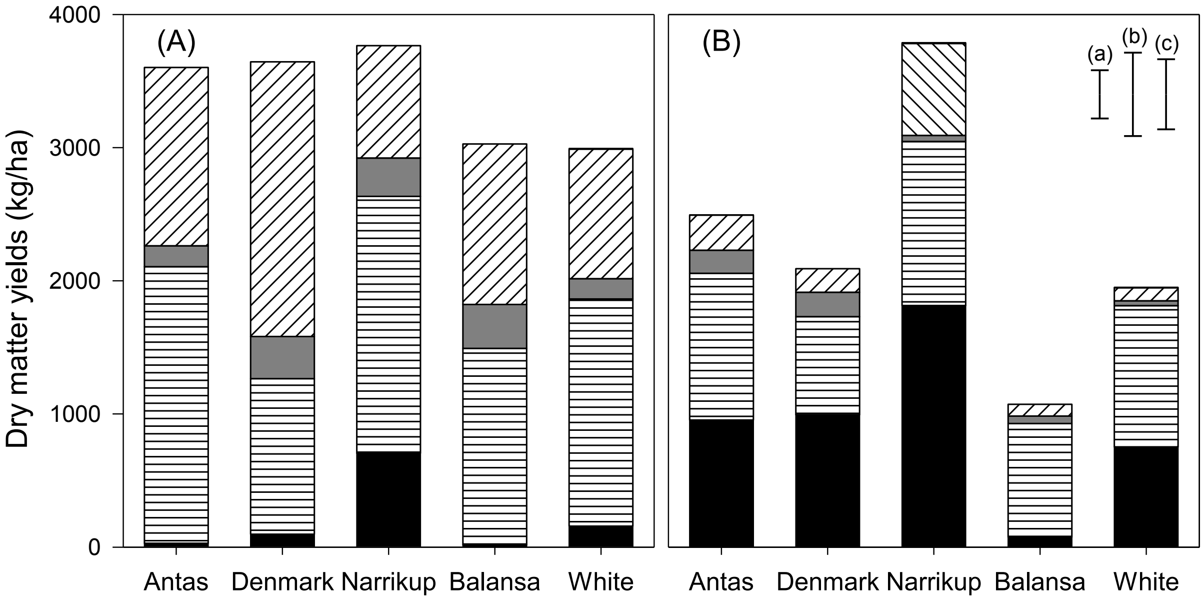

Figure 4.4 Mean dry matter yields and composition for growth of five clover cultivars from 19 September to 27 October 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand for sown clover (■), cocksfoot (▤), broadleaf weed (■), grass weed (▨) and dead matter (▩) components of cultivar plots. (A) is unsprayed, unweeded controls, and (B) is imazethapyr treated plots. Error bars are LSD for main effect of herbicide on (a) sown clover; (b) grass weeds; (c) total dry matter yields.

Plate 4.1 Spinnaker treated 'Narrikup' plot on October 27 2016, with herbicide applied on the 7 April 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand.

4.3.3.3 Total season yields

Total dry matter yields showed a cultivar*herbicide interaction, with only imazethapyr treated 'Narrikup' producing the same total dry matter as its unsprayed unweeded control with 6000 kg DM/ha. Imazethapyr treated 'Antas', 'Denmark', white and balansa clover produced total dry matter yields lower (P=0.021) than their unsprayed unweeded controls (Figure 4.5).

Sown clover yields of 'Narrikup' were higher (P=0.001) than all other cultivars. Imazethapyr treated 'Narrikup' produced 3100 kg DM/ha sown clover, which was greater (P<0.001) than its unsprayed unweeded control with 1500 kg DM/ha. Sown clover yields of imazethapyr treated 'Denmark' and 'Antas' yielded 1250-1275 kg DM/ha, and were greater (P<0.001) than their controls with 120-230 kg DM/ha. White and balansa clover were consistently the lowest (P=0.001) yielding cultivars, and not different to their controls (Figure 4.5).

Sown cocksfoot grass yields were lower (P=0.013) in imazethapyr treatments, with 1300 kg DM/ha, than the unsprayed unweeded controls, with 2800 kg DM/ha (Figure 4.5). This reduction was not consistent for the season, and only an early response to imazethapyr treatment, as yields recovered to be not different in the second harvest (Figure 4.4)

Broadleaf weed yields of imazethapyr treatments were 150 kg DM/ha, less (P=0.002) than their respective controls with 650 kg DM/ha (Figure 4.5).

Grass weed yields were consistently reduced (P=0.006) in imazethapyr treatments with 350 kg DM/ha, and only the annual ryegrass remaining compared to 2400 kg DM/ha in unsprayed unweeded controls where there was also annual brome and phalaris (Figure 4.5). Dead matter yields were lower (P=0.005) in imazethapyr treatments, with 50 kg DM/ha, compared to 300 kg DM/ha in unsprayed unweeded controls (Figure 4.5).

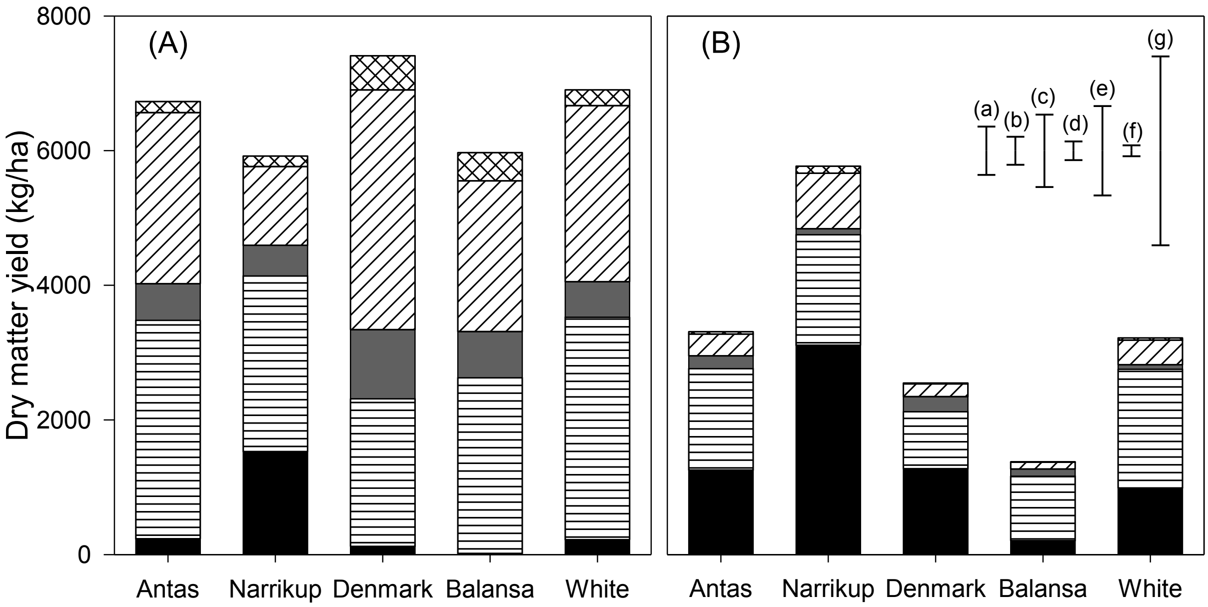

Figure 4.5 Total mean dry matter yields and composition for growth of five clover cultivars from 1 March to 27 October 2016 at Iversen 1, Lincoln University, Canterbury, New Zealand for sown clover (■), cocksfoot (▤), broadleaf weed (■), grass weed (▨) and dead matter (▩) components of cultivar plots. (A) is unsprayed, unweeded controls, and (B) is imazethapyr treated plots. Error bars are LSD for (a) main effect of cultivar on clover yields; (b) main effect of herbicide on clover yields; (c) main effect of herbicide on cocksfoot yield; (d) main effect of herbicide on broadleaf weed yields; (e) main effect of herbicide on grass weed yields; (f) main effect of herbicide on dead matter; (g) cultivar*herbicide interaction for total dry matter yields.

4.4 Discussion

4.4.1 Seedling establishment

The seedling emergence (Figure 3.2) prior to application suggests that most of the sown seed emerged (Table 3.1), and establishment was a success. Any further effects are a result of herbicide applications and environmental impact on persistence.

4.4.2 Imazethapyr + saflufenacil

Based on the results of this experiment, imazethapyr + saflufenacil should not be used as a post-emergence herbicide on subterranean clover/cocksfoot pasture mixes. As in Experiment 1, all clover species showed a rapid phytotoxicity response to the imazethapyr + saflufenacil application with seedling numbers dropping by 80% within 28 days of herbicide application (Figure 4.1 C). Cocksfoot seedlings were also susceptible to this treatment, and at the first harvest in September, all imazethapyr + saflufenacil treated plots were bare. The product also showed a prolonged residual, as any clover seed and weeds that germinated during the experimental period died rapidly after emergence, with only resident weed grasses surviving past emergence by November. The rapid response to treatment application which was observed in the imazethapyr + saflufenacil treatments is attributed to the saflufenacil mechanism of action, which prevents PPO production. PPO inhibition essentially prevents a plant from protecting itself from light, causing rapid necrosis and plant death, which was observed in all treated plots.

4.4.3 Phytotoxicity effects and sown clover yields

In contrast, imazethapyr treated clover seedlings showed no difference in response to the herbicide application relative to their unsprayed unweeded controls (Figure 4.1 A, and B). The sown balansa clover failed to persist after establishing in both the imazethapyr treatment and the unsprayed unweeded control (Figure 4.1 A, and B). Balansa clover is most successful when sown alone or with little competition from existing plants, and seedlings in mixed pastures have a lower chance of survival (Monks & Moot 2010). As the failure to persist was seen in both the imazethapyr treatment, and the unsprayed unweeded control, it is assumed that the balansa clover was unable to compete with the cocksfoot in such dry conditions, despite the elimination of weeds, and should not be advised as the main legume species for a cocksfoot pasture mix. However, it may have been heavily affected by the herbicide, and if it were not, it may have had a better chance after weeds were removed.

EWRS scores showed a difference in response to imazethapyr treatment among cultivars. 'Narrikup' was the only cultivar (Plate 4.1) with a final EWRS score of 3.0, still higher than the unsprayed unweeded control, but well within the commercially acceptable threshold of <5.0 (Figure 4.2 A). This EWRS score was correlated with a September harvest yield which was the same as the unsprayed unweeded control yield, and more than four times higher than all other clover cultivar yields (Figure 4.3). This result suggests that 'Narrikup' was imazethapyr tolerant, with only mild phytotoxicity effects in response to treatment. In contrast, the EWRS phytotoxicity scores indicated that 'Denmark' and balansa clover were affected by imazethapyr treatment. It follows that they can be expected to have significantly reduced dry matter yields relative to their unsprayed unweeded controls. Meanwhile, the lower EWRS scores of 'Antas' and white clover suggest to expect smaller yield reductions. Despite these indications, the early season clover dry matter yields for the four remaining established clovers ('Antas', 'Denmark', balansa, and white clover) were the same with <250 kg DM/ha, and no difference between treated and unsprayed plots (Figure 4.3). This yield was particularly low for such a sowing date, as mid-September yields for subterranean clover of 7000 kg/ha for early March sowing dates have been observed in similar conditions at this site (Moot *et al.* 2003). As these yields were observed in hand weeded, stress free environments, some yield reduction could be expected, but not as significant as was observed. This implies that that the low rainfall in March during establishment, and in April after treatment application had a greater effect on the early growth rate of 'Antas' and 'Denmark' subterranean clover than on 'Narrikup'.

There were no residual effects of imazethapyr treatment on either of the sown pasture components after the September grazing. Post grazing and after one month of recovery, imazethapyr treated 'Narrikup' recovered to again have the highest yield, with 2.5 times more subterranean clover than the unsprayed unweeded control (Figure 4.4). Imazethapyr treated 'Antas' and 'Denmark' recovered to yield 1000 kg DM/ha, confirming the responses found in the literature (Sections 2.6 and 2.7).

White and balansa clover yielded less than 150 kg DM/ha in the September harvest (Figure 3.6), and balansa clover failed to recover later in the season, yielding less than 50 kg/ha in November (Figure 3.7). White clover in the imazethapyr treated plots did recover, yielding 750 kg/ha, but this was still at least 500 kg/ha lower than all the subterranean clover cultivars (Figure 4.5). With the consistently low yields produced by white and balansa clover in this trial, it was shown that they are not ideal options for legume/cocksfoot pasture mixes in these dry autumn environments, and receive no benefit from imazethapyr application.

4.4.4 Cocksfoot

Early season cocksfoot dry matter yields were higher in the unsprayed unweeded control, while the imazethapyr treatments appeared to inhibit early growth of the cocksfoot seedlings, with a 70% reduction in cocksfoot yields (Figure 4.3). However, the cocksfoot recovered from imazethapyr after grazing with no yield differences by 27 October 2016 (Figure 4.4). The effect of imazethapyr on emerging cocksfoot yield has not been fully investigated, but it appears to cause only a temporary yield decrease, with full recovery post grazing. If grazing were to occur earlier, as soon as the imazethapyr treatment withholding period was over (42 DAA), recovery may have been faster, and clover plants may also produce higher yields. Despite cocksfoot being initially checked by imazethapyr, it recovered by late spring, and this means growth is likely to continue through the summer when the subterranean clover sets seed and dies in its annual cycle. This combination allows summer grazing of the pasture. Cocksfoot productivity over the summer is expected to continue to suppress weeds (Mills *et al.* 2014), rather than having the pasture lie fallow and have to mitigate weeds again come the following autumn if a subterranean clover monoculture was sown.

4.4.5 Broadleaf weed control

Imazethapyr reduced broadleaf weed productivity in the early season by >85%, with <50 kg DM/ha remaining (Figure 4.3). Broadleaf weeds showed some recovery after grazing at the second harvest in the imazethapyr treated plots, but were not different to the unsprayed unweeded controls at 200 kg DM/ha (Figure 4.4). It seems likely that interspecific competition for light occurred between the cocksfoot and broadleaf weeds, leading to suppression of the broadleaf weeds due to the dense grass canopy. However, this did not result in higher clover yields in the unsprayed unweeded controls, as the grass also outcompeted the sown clover species.

4.4.6 Grass weed control

Grass weeds were heavily reduced by more than 90% in the imazethapyr treatments (Figure 4.3). These reductions were predominantly the elimination of phalaris and annual brome, but the annual ryegrass was also well controlled, and did not prevent the subterranean clover establishment. Grass weeds remained suppressed by 80% after grazing in the imazethapyr treatments (Figure 4.4). Grass weeds were predominantly annual ryegrass, which is capable of recovering from imazethapyr treatment damage when applied at 60 g ai/ha (Dear *et al.* 2006; Johnson *et al.* 2007), but when growth was suppressed and temperatures were cooler, the cocksfoot and subterranean clover components were able to produce a canopy and dominate. The increased annual ryegrass suppression seen in this experiment may have also been due to a higher imazethapyr application rate, which was 96 g ai/ha (240 g/L imazethapyr treatment at a rate of 400 ml/ha is equivalent to 96 g ai/ha), applied at the early seedling stage when plants had 2-3 leaves. Dear *et al.* (2006) observed full phytotoxicity recovery by 20 DAA when treatment was applied at a rate of 60 g ai/ha. Similarly, Clemmer *et al.* (2004) observed Italian ryegrass suppression of 16-54% when imazethapyr was applied at 75 g ai/ha in the fall when plants were at the 3-4 leaf stage. From these results, and compared with the cocksfoot yields, we can assume that annual grasses are susceptible to imazethapyr when applied at the recommended label rate of 400 ml/ha (in the New Zealand formulation (BASF 2011)) and this susceptibility allows more tolerant, sown species to outcompete the weeds.

4.4.7 Total dry matter yields

Total dry matter yields for the entire growing season showed a cultivar*herbicide interaction, with total yield being dependent on cultivar tolerance of the treatment, as cocksfoot, broadleaf weed, grass weed, and dead matter components yielded the same for all cultivars (Figure 4.5). Imazethapyr treated 'Narrikup' plots at 6000 kg DM/ha were the only sprayed plots not different to their unsprayed unweeded control for total dry matter yields. The composition of the two differed however, with >50% clover DM in the imazethapyr treatment, rather than the 25% in the unsprayed unweeded control. This increase in clover was at the expense of sown cocksfoot (45 reduced to 30%), broadleaf weeds (10 reduced to 2%), and grass weed (20 reduced to 15%) yields. The total DM yields of imazethapyr treated 'Antas' and 'Denmark' were reduced by 50% compared to their unsprayed unweeded controls, but contained 40% sown clover while unsprayed unweeded controls had less than 5% clover content. This reduction in broadleaf and grass weeds in particular creates a higher clover content through increased establishment of subterranean clover in its essential first year. It is likely that this pasture can then set more seed for subsequent years (Smetham, 2003). Even where the cultivar is less tolerant, such as in the case of 'Antas', or has a lower yield potential, as for 'Denmark', the pasture will likely still benefit from an increased seed set due to increased clover content, although this was not evaluated in this study. However, in terms of productivity, a 3000 kg DM/ha yield reduction as was seen in these cultivars is substantial, especially in marginal areas where overall productivity is low, and such a treatment would not be advised if weeds were palatable, or tolerance of the cultivar was uncertain.

Seasonal clover yields were similar to those found in the 'MaxClover' pasture mix grazing experiment carried out by Mills *et al.* (2014), with the sown legume component of a cocksfoot subterranean clover pasture mix yielding 1400-3800 kg DM/ha annually. Cocksfoot yields of this experiment cannot be fully compared, as data were only collected for the subterranean clover growing season. 'Narrikup' produced at the higher end of this range. 'Antas' and 'Denmark' were lower yielding, at 1300 kg DM/ha, but their season would have continued for another two weeks, increasing final yields as flowering had only just begun at the time of harvest. From the second harvest yield, 'Antas' and 'Denmark' were calculated to produce 25-26 kg DM/ha/day, and so with an extra two weeks in the growing season could have yielded at least 350 kg DM/ha more clover, which would place them within the dry matter yields observed in the 'MaxClover' experiments.

Based on the sown clover dry matter yield results, sowing subterranean clover with cocksfoot grass increased the herbicide tolerance of the subterranean clover. When treated with imazethapyr at the 3-4 trifoliate leaf stage, visible phytotoxicity effects were minimized, likely due to decreased herbicide exposure. This treatment also slowed the growth of the sown cocksfoot as the subterranean clover established, and created pastures with a high proportion of subterranean clover in the first year as the subterranean clover took advantage of greater bare ground. However, this is an advantage only for the cultivars that were tolerant, and suited to their environment, as seen by the high 'Narrikup' yields (Figure 4.5). Environmental stress can decrease tolerance, and this happened in this experiment, as was seen in EWRS scores where plants briefly recovered following irrigation (Figure 4.2).

4.5 Conclusions

Imazethapyr treatment increased subterranean clover-cocksfoot pasture yields with no effect on total dry matter yields if the sown cultivar was tolerant like 'Narrikup'. Further to this, broadleaf weeds and weed grasses were controlled at the seedling stage while cocksfoot recovered from an early growth inhibition as a result of the imazethapyr application. Imazethapyr treated 'Narrikup' yielded 6000 kg DM/ha of total dry matter, and half of this was the sown subterranean clover, which would provide high quality lactation feed. While cocksfoot growth was initially reduced, it recovered later in the season to be as productive as the unsprayed cocksfoot, and appeared unaffected heading into the summer season. Even with lower than expected yields, the sown subterranean clover-cocksfoot pasture mixtures benefited from the elimination of broadleaf weed competition, and post-grazing, produced higher yields than the unsprayed unweeded clover components. However these yields may have been better with a less damaging herbicide.

With all subterranean clover cultivars yielding more than 1000 kg DM/ha higher than their unsprayed controls, application of imazethapyr aided in the establishment success of the pasture mix, likely setting the pasture up for success in years to come. This result means imazethapyr can be recommended in mixed establishment scenarios for some subterranean clover cultivars with little adverse effect on the clover or cocksfoot component of the sward.

5 EXPERIMENT 3: PHYTOTOXICITY EFFECTS OF EIGHT HERBICIDE TREATMENTS APPLIED AT THE 1-2 TRIFOLIATE LEAF GROWTH STAGE

5.1 Introduction

Subterranean clovers are particularly vulnerable when germinating in autumn as the emerging seedlings cannot grow as fast as emerging weeds (Evers et al., 1993). Current management protocols are adapted from Australian systems, and there is presently little available information on suitability of commercially available herbicides for subterranean clover in a New Zealand environment. A glasshouse experiment in Australia suggested that application timing and growth stage had little influence on phytotoxic effects on subterranean clover when applied at the 3-4 and 8-10 trifoliate leaf stage (Sandral et al., 1997). However, due to the shortened season in New Zealand, applications can rarely be delayed to the 8-10 leaf stage, and weed control is often required before plants are at the 3-4 leaf stage.

Appropriate management practices in the establishment year for subterranean clover are crucial to ensure persistence in the pasture. High productivity at establishment ensures a high seed set for regeneration in subsequent years (Smetham, 2003). With knowledge of which available herbicides are appropriate for subterranean clover pastures, more comprehensive management protocols can be recommended for farmers looking to use subterranean clover based pastures.

Four recently released subterranean clover cultivars, with at least one from each subspecies, were chosen for comparison against 'Kopu II' as the white clover control. Subterranean clover cultivars were 'Narrikup', a hairy *subterraneum*, 'Denmark', which 'Narrikup' was bred from, 'Antas', with uncertain herbicide tolerance and the only currently available *brachycalycinum* in New Zealand; and 'Monti' a recently introduced *yanninicum*. The effects of herbicides with a range of modes of action (Section 2.5) on establishment, productivity, growth and development, of these subterranean clover cultivars is quantified.

The results from these experiments will fulfil Objective 3 (Figure 1.1), and identify commercially available herbicides suitable for safe application on common sub-clover cultivars in a New Zealand dryland environment at different growth stages.

Chapter 5 reports the effect that herbicides and their application times had on seedling performance of subterranean clovers treated with eight commercially available herbicides when applied at the 1-2 trifoliate leaf growth stage. Chapter 6 reports effects of the same treatments applied at the 4-6 trifoliate leaf stage and discusses differences between the application times.

5.2 Materials and methods

5.2.1 Ashley Dene

Experiments 3 and 4 were sown next to each other in paddock C9 B south (43°38'58.5"S, 172°19'23.8"E, 40 m above sea level), a 1.06 ha paddock on the Lincoln University, Ashley Dene Farm in Springston, Canterbury. The Lismore stony silt loam is very shallow, very stony, and somewhat excessive draining (Hewitt 2010; McLenaghen & Webb 2012). A soil test was performed on 15 March 2016 (Table 3.3) and showed no nutrient deficiencies.

5.2.2 Paddock history

The site has previously been used as part of a series of experiments. In 2004/ spring 2005, 8 x 0.5 ha plots were sown with +/- Caucasian clover (*Trifolium ambiguum*) and tall fescue (*Schedonorus arundinaceus*) mixtures for a grazing trial. These plots were then overdrilled with a 'Karridale'/'Woogenellup' mix. In 2008, these same plots were sown with a 'Woogenellup'/'Campeda' mixture at a rate of 10 kg/ha each with 15 kg/ha of AR37 perennial ryegrass. Resident subterranean clover was present within the trial area, but distinct leaf markings (NSW-DPI 2016) make it easily distinguishable from the sown species. Thus the impact of herbicides on the resident 'Woogenellup' was also assessed. Dominant resident weed species were identified prior to herbicide application, and throughout the experimental period (Table 3.4). Resident grasses were predominantly annual brome weed grasses (*Bromus spp.*), and vulpia hairgrass (*Vulpia spp.*), with minimal remaining tall fescue.

Table 5.1 Common and botanical names of resident broadleaf weed species in C9BS paddock, Ashley Dene farm, Springston, Canterbury, New Zealand.

Common name	Botanical name
Chickweed	*Stellaria media* (L.) Vill.
Fathen	*Chenopodium album* L.
Shepherds Purse	*Capsella bursa-pastoris* (L.) Medik.
Scrambling Speedwell	*Veronica persica* Poir.
Musky Storksbill	*Erodium moschatum* (L.) L'Hér.

5.2.3 Meterological conditions

Autumn rainfall at Ashley Dene was well below average with a cumulative 52.8 mm from February – April 2016 (Figure 5.1) compared to the LTM of 150 mm for the same period (Table **3.2**). This low rainfall combined with high air temperatures of 15-18˚C and 2 cm depth soil temperatures of 19-22˚C (Figure 5.1) delayed the beginning of the field trial. Rainfall for the sown trial period (March – November 2016) was a cumulative 347 mm (Figure 5.1), 141 mm less than the LTM of 488 mm (Table **3.2**).

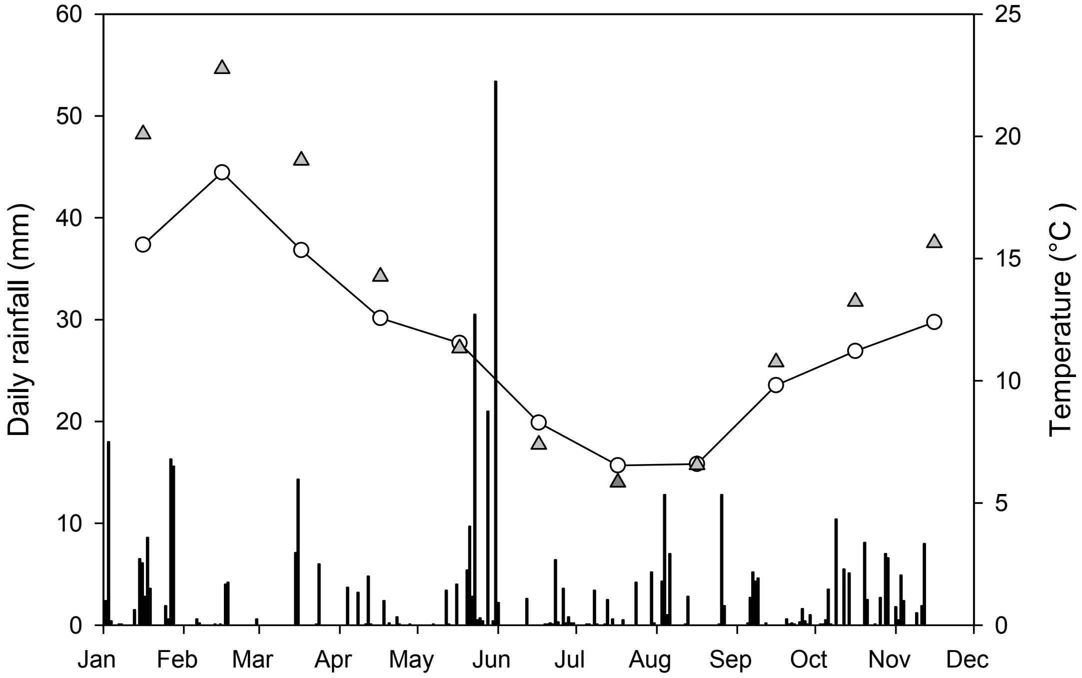

Figure 5.1 Daily rainfall (■) and mean monthly air (○) and soil temperatures at 2 cm depth (▲) for January - November 2016 from ADC8 weather station for Ashley Dene. Rainfall is daily rainfall (mm); soil and air temperatures (°C) are monthly means.

5.2.4 Site preparation

The site was sprayed on 22.02.2016 with 5 L/ha Buster herbicide (a.i. 200 g/l glufosinate-ammonium) in 200 L water. A second application occurred on the 14.03.2016 to control the resident weed species (Table 5.1) prior to sowing.

5.2.5 Experimental design

For Experiment 3, five 30 m strips of subterranean clover cultivars, two drill rows wide (4.2 m) with three replicates were drilled in a randomized strip-plot design for a post-emergence herbicide application experiment. For Experiment 4, a second set of plots was replicated directly adjacent with a 2.1 m buffer zone for application at a later (4+ trifoliate leaf) development stage. Unsprayed seed of subterranean clovers ('Antas', 'Narrikup', 'Monti' and 'Denmark') were sown at a rate of 20 kg/ha, and white clover ('Kopu II') controls at 5 kg/ha. Germination data for these cultivars can be found in Section 3.2.1 (Table 3.1). Seed was drilled into both experiments on 22.03.2016 using a Flexiseeder drill at a target depth of 3 mm, with drill widths of 150 mm, and then rolled with a heavy roller.

5.2.6 Herbicide application

The eight herbicide treatments, (Table 5.2) were applied in strips at 90° across sown clover strips. These were randomised for each replicate, to create two split-strip plot experimental designs (Montgomery 2013). Two unsprayed strips were left in each replicate as unsprayed unweeded controls. Herbicide applications were carried out with a CO_2 pressurized backpack sprayer with a tank capacity of 6 L, and an attached 2 metre centre hold precision boom with four V110 04 AC nozzles. The sprayer was calibrated to put out a constant 800 ml per nozzle per minute, applied at a constant walking speed of 5 km/h. Herbicide mixtures were made up in 4 L quantities. All treatments were applied at their recommended label rate with Hasten™ as an adjuvant to remove permeability concerns, except flumetsulam, which has its own wetting agent within its formulation.

Table 5.2 Herbicide trade names, active ingredient, mode of action, and rate applied for Experiments 3 and 4. Alternative tradenames and commercial availability can be found in the appendix (Table A.2).

Herbicide	Active Ingredient	Mode of Action	Product rate L/ha
Dow AgroSciences 2,4-DB	400 g/L 2,4-DB	Auxin-type action	8.0
Dow AgroSciences MCPB	385 g/L MCPB	Auxin-type action	7.5
Basagran	480 g/L bentazone	Photosynthesis inhibition	3.0
Headstart	50 g/L flumetsulam	ALS inhibition	1.0
Spinnaker	240 g/L imazethapyr	ALS inhibition	0.4
Weedmaster360	360 g/L glyphosate	Amino acid biosynthesis inhibition	1.0
Jaguar	250 g/L bromoxynil + 25 g/L diflufenican	Photosynthesis inhibition	1.0
Pulsar	200 g/L MCPB + 200 g/L bentazone	Auxin-type action + Photosynthesis inhibition	6.0

The application of herbicide for Experiment 3 occurred at the 1-2 trifoliate leaf stage, on the 14.06.16, almost three months after sowing due to no rainfall until 20 May (Figure 5.1). Wind direction was a prevailing northerly with average speeds of 9-11 km/h for the duration of application. Average air temperature was 9.6 ˚C, and average 2 cm depth soil temperature was 6.7 ˚C.

Application for Experiment 4 was delayed until the 12.07.16, when subterranean clover seedlings had 4+ trifoliate leaves. Wind direction was a prevailing northerly with average speeds of 10-12 km/h for the duration of application. Average air temperature was 6.6 ˚C, and average soil temperature at 2 cm depth was 4.7 ˚C.

5.2.7 Measurements

Measurements of seedling establishment, phytotoxicity, and herbage dry matter production were carried out as in Section 3.2.9, unless otherwise stated.

5.2.7.1 Plant development

At harvest, average development stages of each plot were recorded, and three plants from each plot were dug up and measurements for plant development stage, burr/flower number, root length, plant height, diameter, leaf width taken. These individual plants were also scored for visible herbicide damage.

Development stages were defined simply, from Thomas (2003). Trifoliate leaves were counted until there were >10, at which point axillary buds (nodes) on primary stems (runners), and number of runners per plant were counted. When flowering began, the number of reproductive buds, open flowers, and developing seed heads and mature seed heads (stage 3 burrs) per runner were counted.

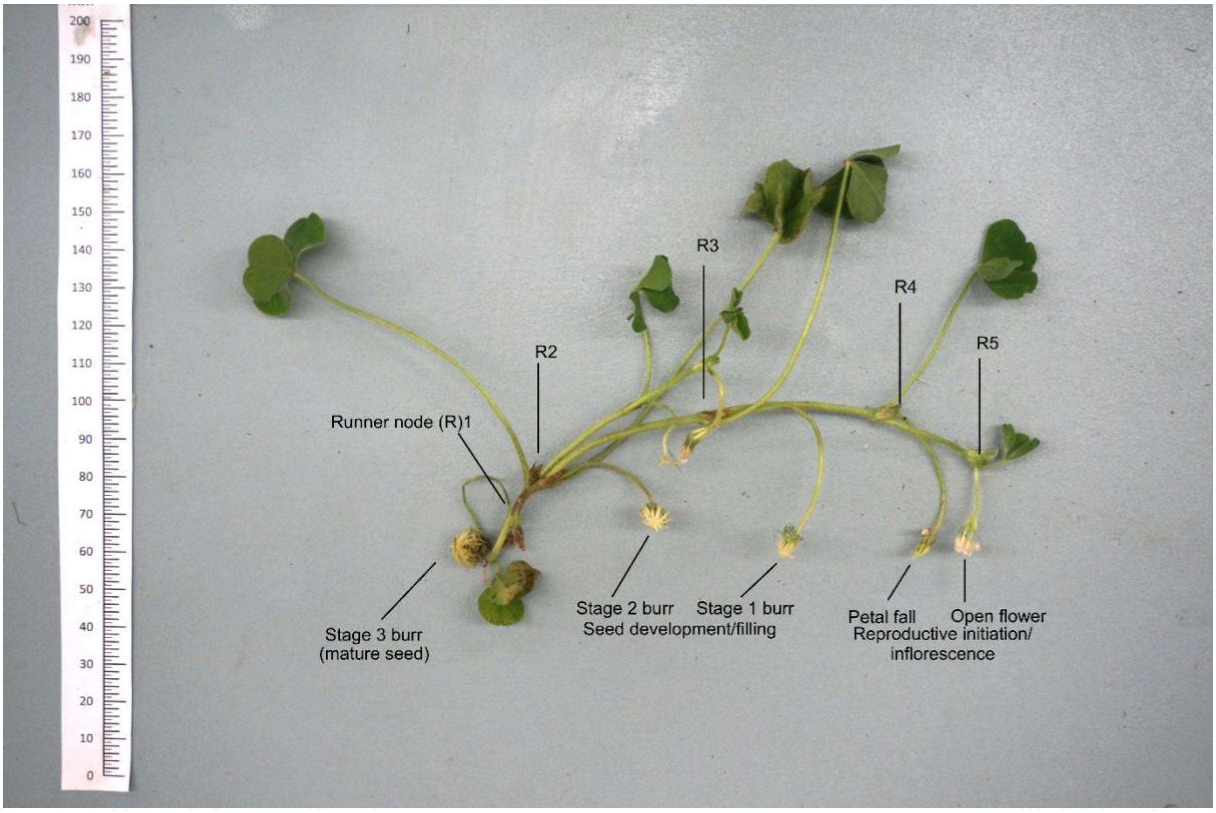

Plate 5.1 Simple subterranean clover development stages, adapted from Nori (2013); Thomas (2003).

5.2.7.2 Dry matter harvests

For both experiments, plots were harvested twice, the first harvest from the 16-21 September 2016, and the second from the 8-10 November 2016, after 40 days regrowth post-grazing and topping.

Plots treated with glyphosate were bare in both Experiments 3 and 4, with an EWRS score of 9.0 at the time of the first harvest. They were eliminated from physical harvesting, and recorded as a negative control.

5.2.8 Agronomic management

Immediately following the first harvest from September 23 until September 30, the plots were grazed by a mob of ewes and new season lambs. The site was then topped with a Fieldmaster TM twin belt drive pasture topper to remove the remaining seed heads from ungrazed weeds. The site was also heavily grazed again after the second harvest, on the 14.11.2016.

5.2.8.1 Long-term weed control

Residual weed control was visually assessed on the 15.01.2017, with treatments assigned a score from 1-10 of approximate weed proportion compared to the unsprayed unweeded controls. Where 1 = <10% and 10 = 100% of broadleaf weed recovery compared with controls.

5.2.9 Statistical analysis

Statistical analyses were carried out using Genstat v. 16. Figures were produced in SigmaPlot v. 11.

Seedling establishment was analysed using a two-way ANOVA of cultivar by herbicide for all plots to establish any differences among cultivars prior to herbicide application. Comparisons of herbicide treatment and timing effects on seedling establishment, development, EWRS scores, DM production and botanical composition of cultivar plots were performed using a two-way split strip-plot design. ANOVAS had a nested treatment structure of cultivar*herbicide, using reps as blocking factors, herbicide strips as rows, and cultivar strips as columns.

Plant diameter was analysed using a two-way ANOVA of cultivar by herbicide. Development stages were analysed in September as the number of nodes (axillary buds) per runner. In November, number of runners per plant, number of reproductive nodes per plant (quantified as total reproductive count per runner multiplied by number of runners per plant), and number of mature seed carrying burrs per plant (number of stage 3 burrs per runner multiplied by number of runners per plant), were analysed to provide an indication of the effect of herbicide on reproductive success.

Means separations were carried out using Fisher's unprotected Least Significant Difference tests at $\alpha = 0.05$.

5.3 Results

5.3.1 Seedling establishment

There was no difference in seedling establishment prior to treatment application on Experiment 3 among cultivars (P=0.178), herbicides (P=0.544), or experiments (P=0.954) with an average of 136 ± 10 plants per m^2.

5.3.2 Phytotoxicity assessment

EWRS phytotoxicity scores of subterranean clover plants treated with herbicide at the 1-2 trifoliate leaf stage showed only a primary effect of herbicide, and no differences in response among white clover and subterranean clover cultivars. Average EWRS scores of treatments increased (P<0.001) compared to unsprayed unweeded controls over time. By 7 DAA, all treatments except flumetsulam and bromoxynil + diflufenican were different (P<0.001) to their controls, and glyphosate had exceeded the commercially acceptable EWRS score threshold (Figure 5.2). EWRS phytotoxicity scores for all treatments at 28 DAA were also different (P<0.001) to their controls, but not different to their 7 DAA scores, except for glyphosate.

By 63 DAA, bentazone treated clover plots had recovered to be no different to their control. All other treatments had EWRS scores greater (P<0.001) than their controls. Glyphosate was the highest scoring with 8.6, and different to all other treatments (P<0.001), followed by 2,4-DB with a score of 4.8, which was just within the commercially acceptable EWRS score threshold of 5.0 (Figure 5.2). All other treatments ranged from 2.4-3.4, and were higher (P<0.001) than their unsprayed unweeded controls, but within the commercially acceptable threshold (Figure 5.2). Visible leaf phytotoxicity symptoms of 'Narrikup' subterranean clover for all treatments can be seen in Plate 5.2, as an example of the typical symptoms shown across cultivars.

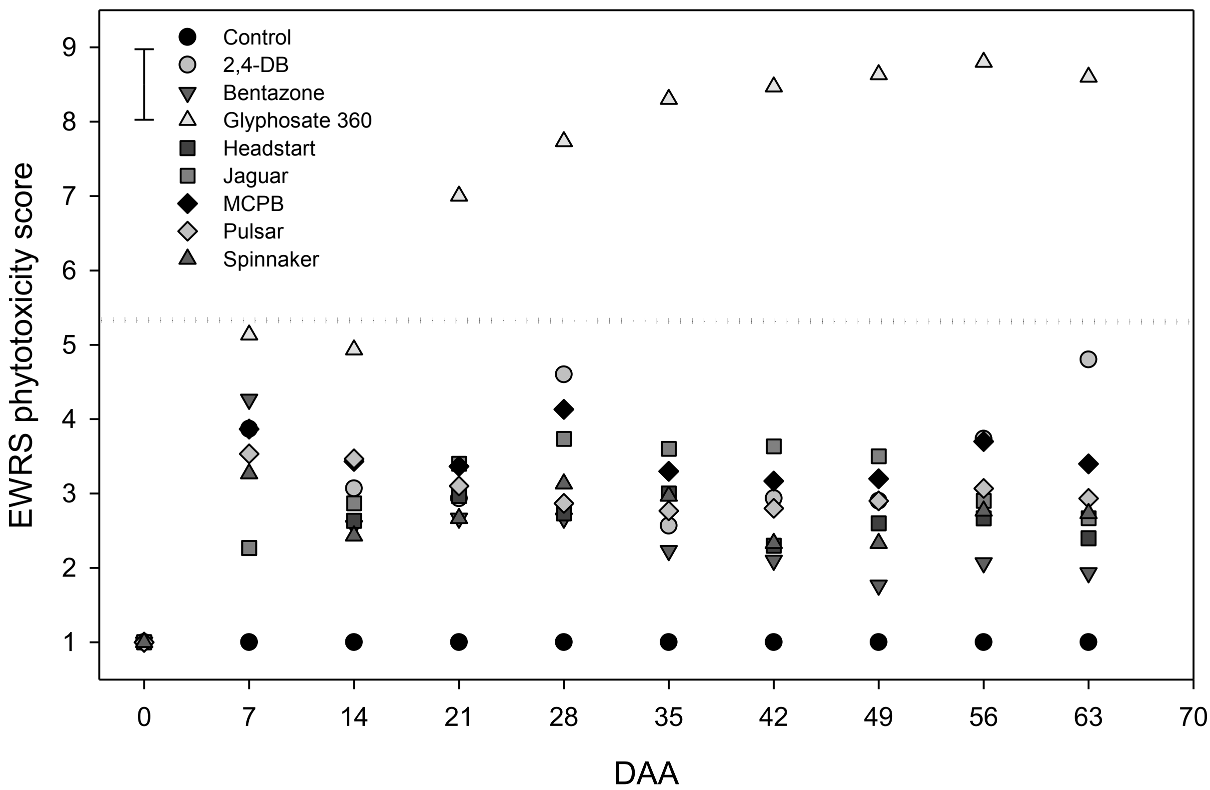

Figure 5.2 Mean EWRS phytotoxicity scores for nine herbicide treatments from the 13 June – 9 August 2017 at Ashley Dene, Springston, Canterbury, New Zealand after application on the 14 June 2017. Error bar is LSD for herbicide*time interaction.

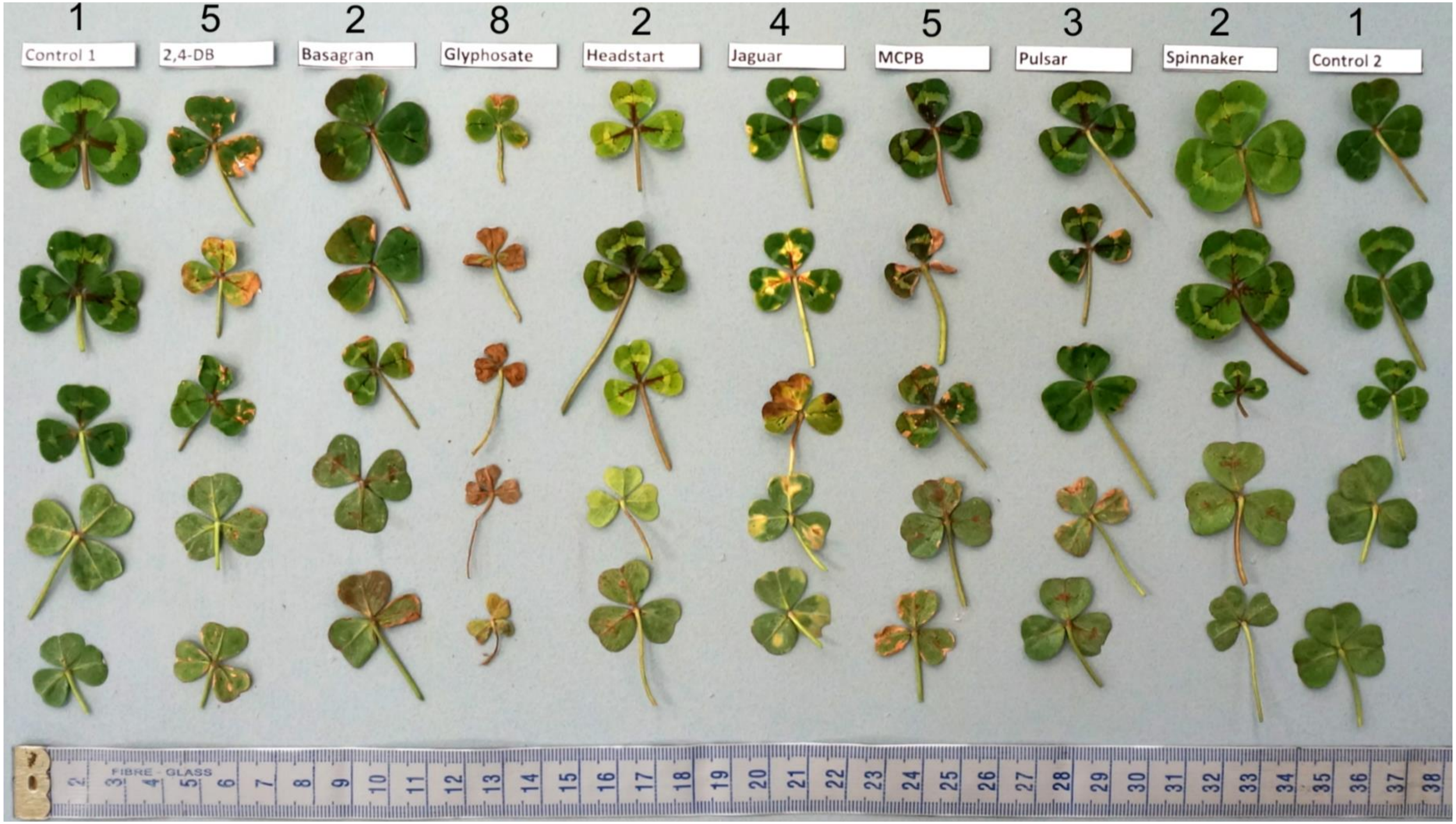

Plate 5.2 Typical leaf phytotoxicity symptoms of subterranean clover cultivar 'Narrikup' to all herbicide treatments 30 DAA, on the 14 July 2016. Numbers indicate EWRS score for treatments applied at the 1-2 trifoliate leaf stage on 14 June 2016 at Ashley Dene, Springston, Canterbury, New Zealand. Photos for other cultivars can be found in the appendix (Plate A.1, Plate **A.2**, and Plate **A.3**).

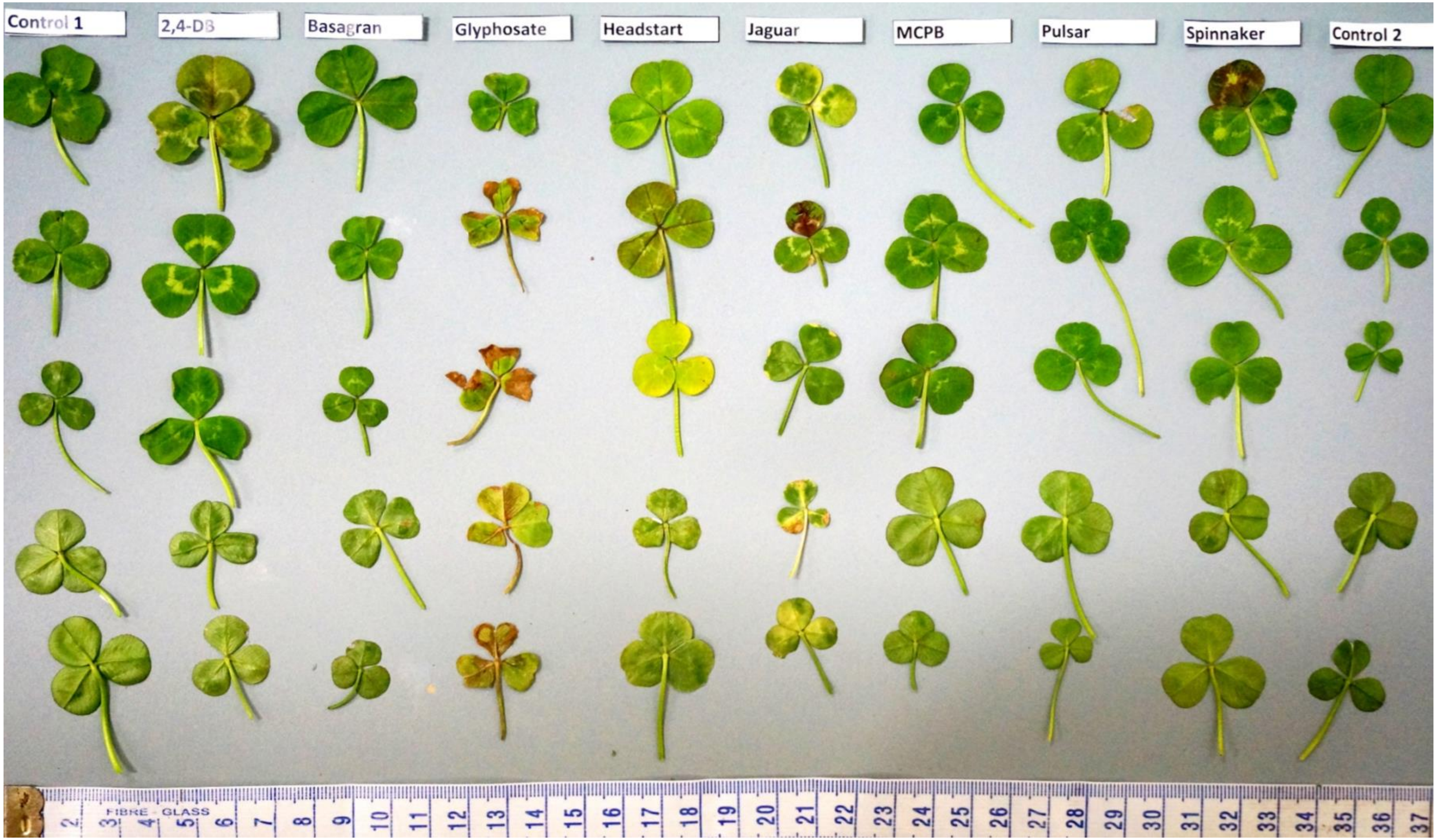

Plate 5.3 Difference in leaf phytotoxicity symptoms of white clover cultivar 'Huia' to all herbicide treatments 30 DAA, on the 14 July 2016 at Ashley Dene, Springston, Canterbury, New Zealand.

5.3.3 Harvest 1: 16 September 2016

There was an interaction between sown clover yields and herbicide (P<0.001) as shown in Figure 5.3. Sown clover yields were highest (P<0.001) for 'Narrikup' and 'Antas' unsprayed unweeded controls, flumetsulam treated 'Narrikup, and bentazone treated 'Antas', yielding 450-550 kg DM/ha.

Sown clover yields for all 'Narrikup' herbicide treatments yielded the same as controls with 250-500 kg DM/ha, except for (P<0.001); 2,4-DB treated 'Narrikup with 150 kg DM/ha, and MCPB sprayed 'Narrikup' with 200 kg DM/ha.

Sown clover yields for all herbicides on 'Antas' except bentazone were lower (P<0.001) than the unsprayed unweeded control, producing <300 kg DM/ha.

Sown clover yields for all remaining subterranean clover cultivars and were not different to their controls, producing <100 kg DM/ha, under all herbicides.

White clover yields for all herbicides were not different to their unsprayed unweeded controls, with <100 kg DM/ha.

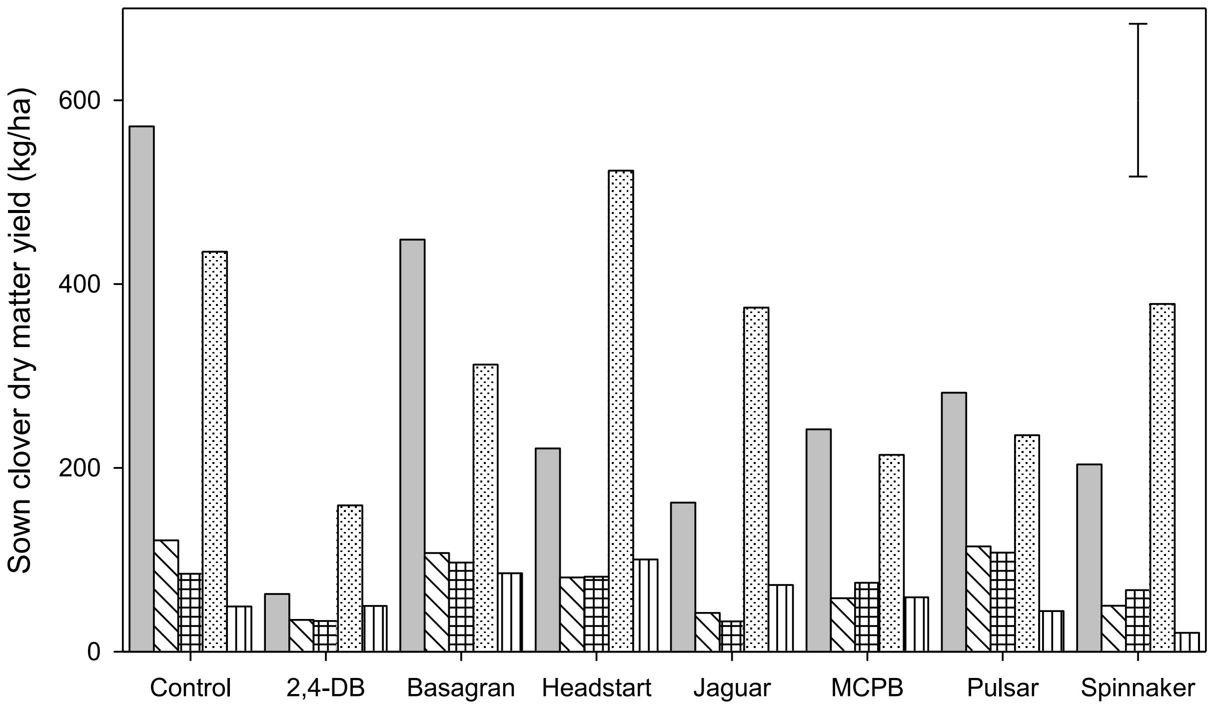

Figure 5.3 Mean sown clover dry matter yields of 'Antas' ( ) 'Denmark' ( ), 'Monti' ( ), 'Narrikup' ( ), white clover ( ), for all treatments in Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand. Harvested 16 September 2016. Error bar is LSD for cultivar*herbicide interaction.

There was no evidence of a sown cultivar*herbicide interaction for yields of resident 'Woogenellup' clover (P=0.593), broadleaf weeds (P=0.146), grass weeds (P=0.122), or dead matter (P=0.629).

There was a main effect of herbicide on resident clover yields. Resident clover ('Woogenellup') yields were highest (P=0.004) in bentazone plots with 200 kg DM/ha, and not different to the unsprayed unweeded control yields of 150 kg DM/ha. 2,4-DB, imazethapyr, and bromoxynil + diflufenican reduced resident clover yields compared with controls, with <100 kg DM/ha. All remaining herbicides were not different to their respective unsprayed unweeded controls.

Broadleaf weed yields were highest in the unsprayed unweeded controls, with 1000 kg DM/ha, and lowest (P=0.001) in flumetsulam, imazethapyr and bromoxynil + diflufenican, with <100 kg DM/ha. Broadleaf weed yields for all other herbicides were reduced (P=0.001) compared with their unsprayed unweeded controls, with 300-500 kg DM/ha. Grass weed yields were highest (P=0.031) in bromoxynil + diflufenican plots, with 250 kg DM/ha. All other herbicides had grass weed yields <150 kg DM/ha, and were not different to controls. Dead matter represented <25 kg DM/ha in all herbicides (Figure 5.4). Total dry matter yields were higher (P<0.001) in the unsprayed unweeded controls at 1600 kg DM/ha than all other herbicide treatments with <900 kg DM/ha (Figure 5.4).

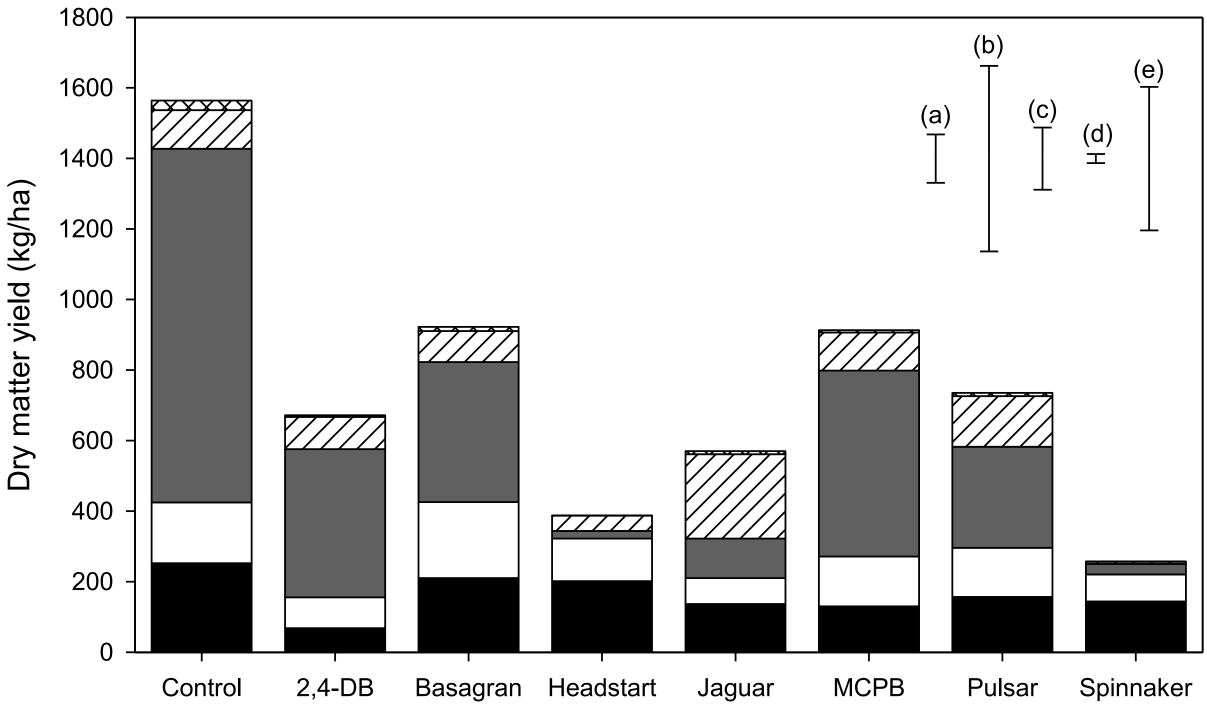

Figure 5.4 Mean dry matter yields and composition for herbicide treatments of Experiment 3, applied at the 1-2 trifoliate leaf stage on 14 June 2016, harvested 16 September 2016 at Ashley Dene, Springston, Canterbury, New Zealand. Categories are sown clover (■), resident clover (□), broadleaf weed (■), grass weed (▨) and dead matter (⊠). Error bar is LSD for main effect of herbicide on (a) resident subterranean clover; (b) broadleaf weeds; (c) grass weeds; (d) dead matter; (e) total dry matter yields.

5.3.4 Harvest 2: 10 November 2016

There was a sown clover*herbicide interaction for sown clover yields. Sown clover yields of imazethapyr treated 'Narrikup' were higher (P=0.042) than all other cultivars and herbicide treatments (Figure 5.5).

Sown clover yields of bentazone, 2,4-DB, and bentazone + MCPB treated 'Narrikup' were not different to their unsprayed unweeded control, with 550-1050 kg DM/ha. Bromoxynil + diflufenican, flumetsulam, and MCPB sprayed 'Narrikup' produced higher (P=0.042) sown clover yields than their unsprayed unweeded control, with 1200-1400 kg DM/ha.

Sown clover yields for imazethapyr over 'Antas' at 1550 kg DM/ha were greater (P=0.042) than the unsprayed unweeded control, which had 850 kg DM/ha . All remaining herbicide treatments for 'Antas' were not different to their unsprayed unweeded controls, with 500-1400 kg DM/ha.

Sown clover yields of all remaining cultivars and herbicides were not different to their controls, and <750 kg DM/ha (Figure 5.5).

White clover yields were <550 kg DM/ha for all herbicides and the unsprayed unweeded control.

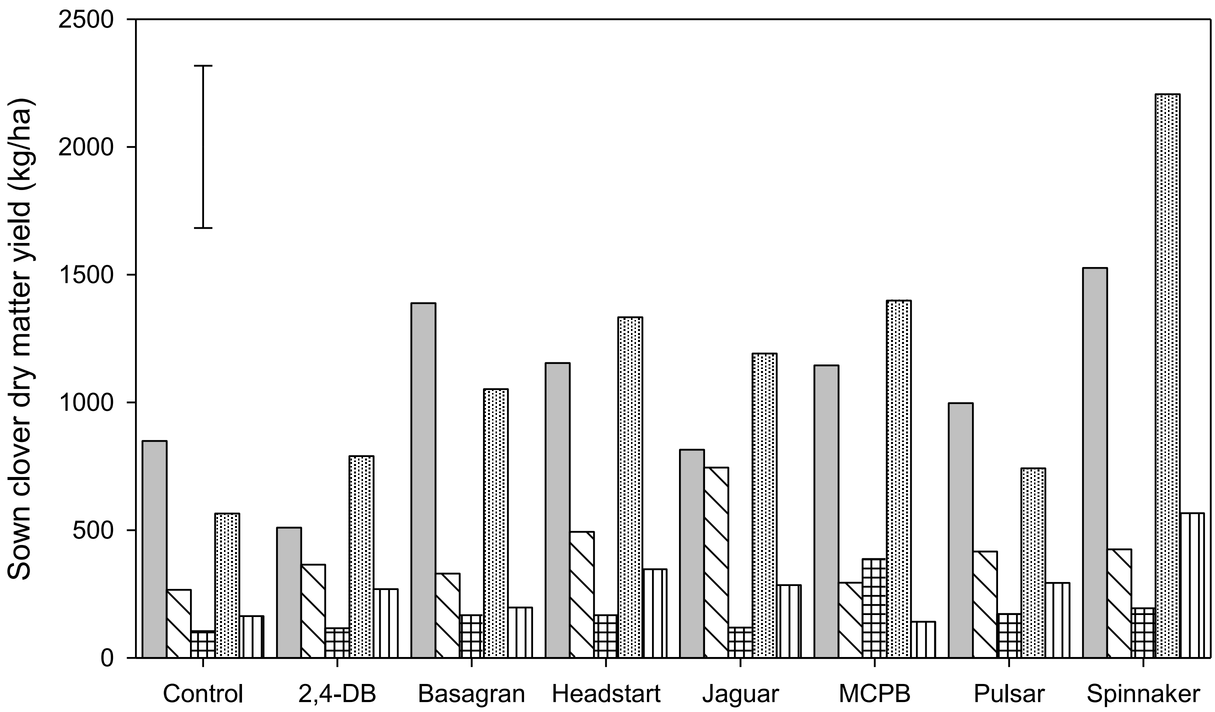

Figure 5.5 Mean sown clover dry matter yields of 'Antas' ( ) 'Denmark' ( ), 'Monti' ( ), 'Narrikup' ( ), white clover ( ), for all herbicide treatments in Experiment 3 from 26 September to 10 November 2016 with herbicides applied at the 1-2 trifoliate leaf stage on 14 June 2016 at Ashley Dene, Springston, Canterbury, New Zealand. Harvested 10 November 2016. Error bar is LSD for cultivar*herbicide interaction.

There was no evidence of a sown cultivar*herbicide interaction for yields of resident 'Woogenellup' clover (P=0.649), broadleaf weeds (P=0.288), grass weeds (P=0.172), or dead matter (P=0.700). Total dry matter yields were not different (P=0.133) to the unsprayed unweeded controls at 2500 kg DM/ha.

Resident clover yields were the same (P=0.129), at 500-1000 kg DM/ha for all treatments. Combined sown + resident clover yields were highest in imazethapyr, flumetsulam, bentazone and bromoxynil + diflufenican treatments, at 1500-2100 kg DM/ha. All other herbicide treatments were not different to the unsprayed unweeded control at 900 kg DM/ha.

As expected, broadleaf weed yields were highest (P<0.001) in the unsprayed unweeded controls with 1040 kg DM/ha. Broadleaf weed yields were lowest (P<0.001) in imazethapyr and flumetsulam treatments, with 75 kg DM/ha. Broadleaf weed yields for all other treatments were reduced (P<0.001) compared with controls, with 200-350 kg DM/ha (Figure 5.6). Grass weed yields were higher (P=0.023) in 2,4-DB treatments with 1050 kg DM/ha than the controls with grass weed yields of 550 kg DM/ha. All other herbicide treatments yielded <1000 kg DM/ha of grass weeds, and were not different to the controls (Figure 5.6). Dead matter yields were not different (P=0.432) for all treatments, with 75-180 kg DM/ha (Figure 5.6).

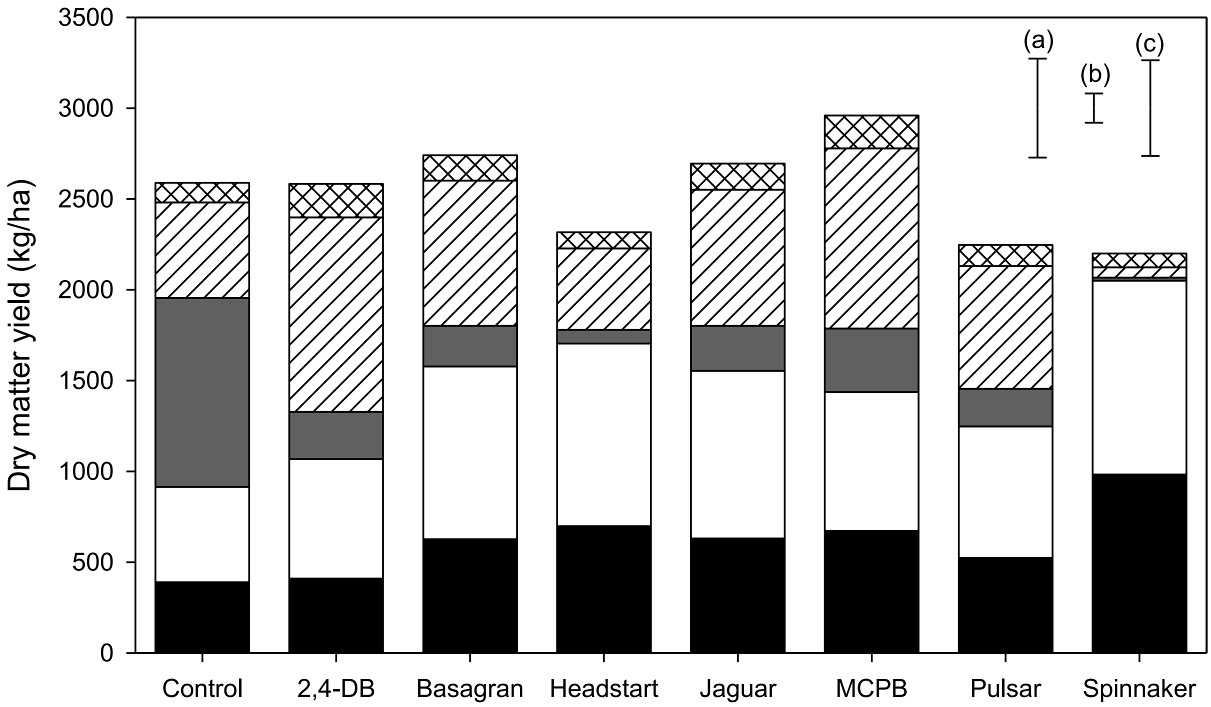

Figure 5.6 Mean dry matter yields and composition for herbicide treatments of Experiment 3, from 26 September to 10 November 2016 with applications at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand. Categories are sown clover (■), resident clover (□), broadleaf weed (■), grass weed (▨) and dead matter (⊠). Error bar is LSD for main effect of herbicide on (a) combined (sown+resident) clover; (b) broadleaf weeds; (c) grass weeds.

5.3.5 Total season harvest yields

The combined 2016 season harvest data had a cultivar*herbicide interaction (P=0.003), and sown clover yields were highest (P=0.003) in imazethapyr treated 'Narrikup', with 2600 kg/ha (Figure 5.7, Plate 5.4).

Sown clover yields of 1850 kg DM/ha for flumetsulam treated 'Narrikup' were also higher (P=0.003) than its control, with 1000 kg DM/ha. Sown clover yields for all remaining treated 'Narrikup' plots were the same as the control, with 950-1600 kg DM/ha.

Sown clover yields for 'Antas' were not different to their control, with 1000-1850 kg DM/ha, except for a reduction (P=0.003) in 2,4-DB treated 'Antas', at 550 kg DM/ha.

All remaining cultivars and treatments were not different to their controls, or one another (Figure 5.7).

White clover yielded <600 kg DM/ha for all herbicides and the unsprayed unweeded control.

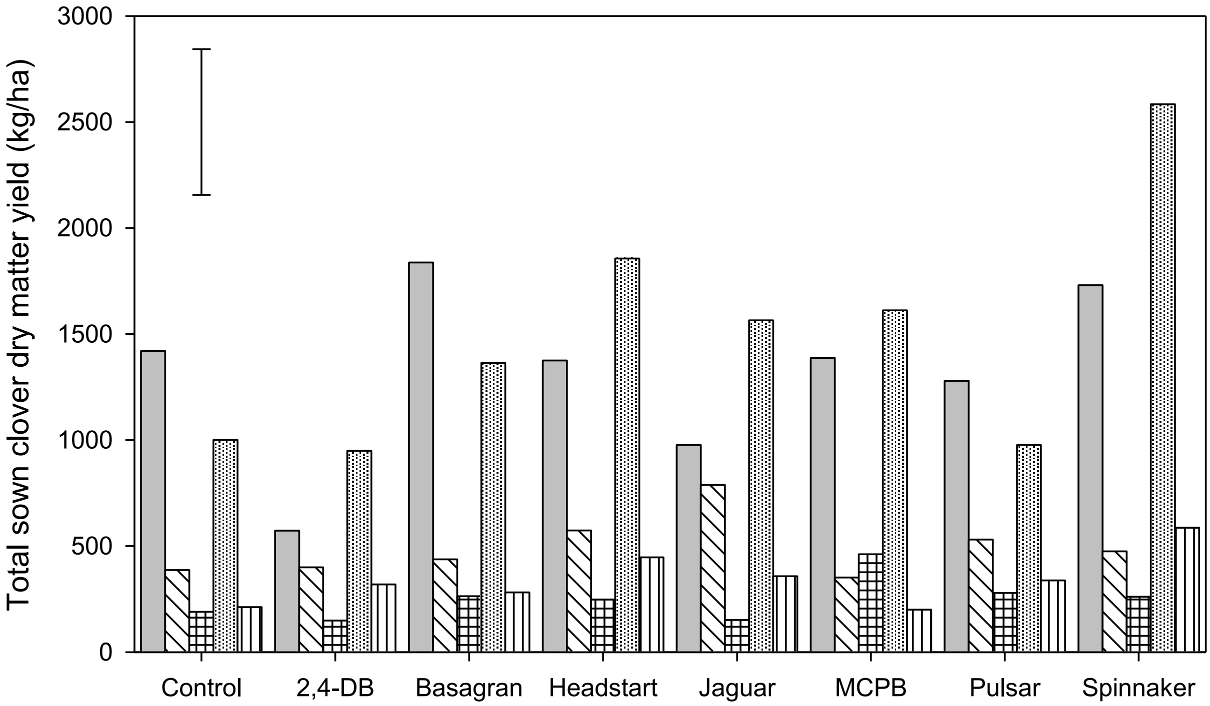

Figure 5.7 Total sown clover dry matter yields for all treatments of 'Antas' ( ) 'Denmark' ( ), 'Monti' ( ), 'Narrikup' ( ), white clover ( ), in Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand from 22 March to 10 November 2016. Error bar is LSD for cultivar*herbicide interaction.

There was no evidence of a sown cultivar*herbicide interaction for yields of resident 'Woogenellup' clover (P=0.638), combined (sown+resident) clover (P=0.190) broadleaf weeds (P=0.196), grass weeds (P=0.184), or dead matter (P=0.620). Resident clover yields were not different (P=0.180) among treatments, with a mean 950 kg DM/ha.

Mean combined (sown + resident) clover yields for imazethapyr, flumetsulam, and bentazone treatments, were 2000-2300 kg DM/ha clover and higher (P=0.019) than the unsprayed unweeded control, and 2,4-DB treatments, which yielded 1200-1300 kg DM/ha clover. All other treatments were not different to the unsprayed unweeded controls, at 1500-1800 kg DM/ha clover.

As expected, broadleaf weed yields were highest in the unsprayed unweeded controls, with 2000 kg DM/ha, and greater (P<0.001). Bromoxynil + diflufenican, flumetsulam, and imazethapyr had the lowest (P<0.001) broadleaf weed yields, with <350 kg DM/ha. Broadleaf weed yields of remaining treatments were reduced (P<0.001) compared to the control, with 500-900 kg DM/ha.

Grass weed yields were highest in 2,4-DB and MCPB treatments, with 1100-1200 kg DM/ha, but not different to controls, which yielded 650 kg DM/ha. Grass weed yields for flumetsulam and imazethapyr treatments, while not different to controls, were reduced (P=0.025) compared to 2,4-DB and MCPB treatments. Dead matter yields were minimal at 100-200 kg DM/ha (Figure 5.8).

Total dry matter yields were highest in the unsprayed unweeded controls at 4150 kg DM/ha (Figure 5.8). MCPB and bentazone were not different to this. All other herbicides yielded lower than the unsprayed unweeded controls, with 2450-3250 kg DM/ha.

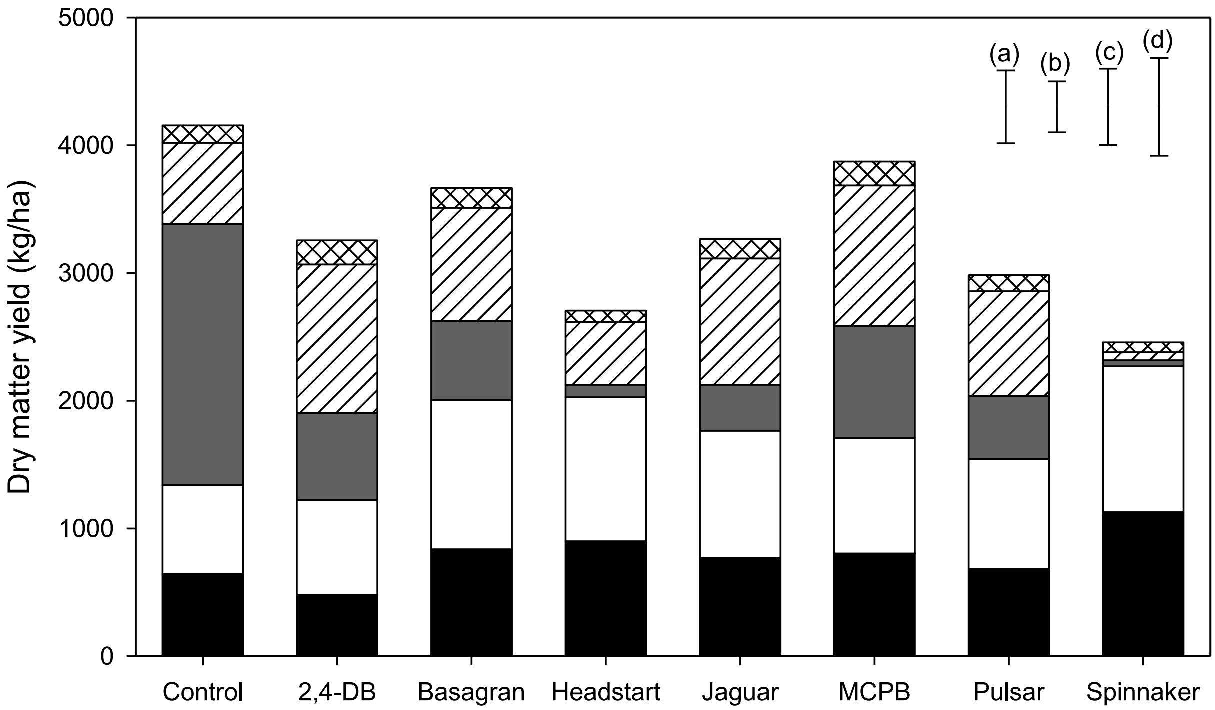

Figure 5.8 Total mean dry matter yields for growth from 22 March to 10 November 2016 for all categories for herbicide treatments of Experiment 3, applied at the 1-2 trifoliate leaf stage on 14 June 2016 at Ashley Dene, Springston, Canterbury, New Zealand. Categories are sown clover (■), resident clover (□), broadleaf weed (■), grass weeds (▨) and dead matter (⊠). Error bar is LSD for main effect of herbicide on (a) combined (resident+sown) clover; (b) broadleaf weeds; (c) grass weeds; (d) dry matter.

Plate 5.4 Imazethapyr treated clover between lines, next to glyphosate treated on the right, and bentazone treated on the left, at Ashley Dene, Springston, Canterbury, New Zealand on the 11.11.2016, six months post herbicide treatment at the 1-2 trifoliate leaf stage on the 14 June 2016.

5.3.6 Plant development

5.3.6.1 September 2016:

There was a cultivar*herbicide interaction (P=0.002) for plant diameter in the September 2016 harvest (Table 5.3). 'Antas' showed little response in plant diameter to treatment, with unsprayed plants measuring 217 ± 28 mm across, but 2,4-DB treated plants were reduced to 92 ± 28 mm.

Unsprayed 'Denmark' was the smallest plant, at 18 ± 28 mm, and only 2,4-DB, MCPB and imazethapyr treatments had no increase in plant diameter. Remaining treatments were larger (P=0.002) than the unsprayed control, but not different to each other, at 114-170 cm in diameter.

Unsprayed 'Narrikup' was the largest in diameter, at 313 ± 28 mm across, and bentazone, flumetsulam, bromoxynil + diflufenican, and bentazone + MCPB treatments were not different. 2,4-DB treated 'Narrikup' was smaller than all other treatments, at 85 ± 28 mm. Imazethapyr and MCPB treated Narrikup at 149-222 mm were smaller than the control, but larger than 2,4-DB treated Narrikup®.

White clover showed no response in plant diameter as a result of herbicide treatment, with unsprayed plants averaging 81 ± 28 mm (Table 5.3).

Table 5.3 Mean sown clover plant diameter on September 23 2016 for all treatments of subterranean clover in Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand at 23 September 2016. * indicates different to control for cultivar*herbicide interaction (P=0.002, e.s.e.=28).

Treatment	Antas	Denmark	Monti	Narrikup	White Clover
Control	217	18	56	313	81
2,4-DB	92 *	82	27	85 *	111
Basagran	233	143 *	102	263	55
Headstart	249	170 *	118	264	140
Jaguar	208	145 *	156 *	291	100
MCPB	205	60	104	149 *	97
Pulsar	185	114 *	95	293	54
Spinnaker	162	87	46	222 *	44

There was no cultivar*herbicide interaction for the average number of nodes per runner for sown subterranean clover at the time of the first harvest on 23 September 2016, only main effects of cultivar (P<0.001) and herbicide (P<0.001) treatment showed differences. When measured on the 23 September 2016, 'Narrikup' was the most developed (P<0.001), with five nodes per runner compared to all other cultivars. 'Antas' and 'Denmark' both had four nodes per runner, while 'Monti' was the least advanced (P<0.001), with three nodes per runner.

Plant development was delayed (P<0.001) by 2,4-DB. These plants had only two nodes per runner, compared to all other treatments with 3-4 nodes per runner (Figure 5.9). There was no difference in development stage among remaining treatments and the unsprayed unweeded controls, showing no effect of herbicide on subterranean clover development.

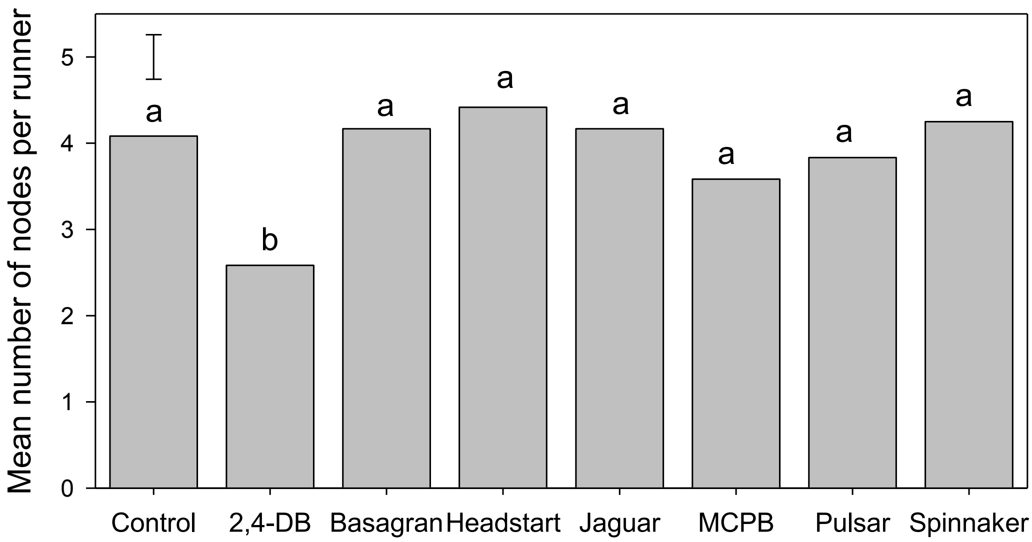

Figure 5.9 Mean number of nodes per runner of all treatments in Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand at 23 September 2016. Error bar is LSD for main effect of herbicide. Bars with a letter in common are not significantly different at α=0.05.

5.3.6.2 November 2016:

There was also no cultivar*herbicide interaction for plant diameter after grazing and recovery at the November harvest, only primary effects of cultivar (P<0.001) and treatments (P<0.001). 'Narrikup', 'Woogenellup', and 'Antas' at 345-410 mm in mean diameter were larger (P<0.001) than 'Denmark' at 190 mm diameter.

Unsprayed subterranean clover plants were larger (P<0.001) than all treated plants, at a mean 480 mm diameter (Figure 5.10, Plate 5.5). 2,4-DB treated plants were the smallest (P<0.001) at a mean diameter of 165 mm. All remaining treatments were not different to each other, at 310-365 mm in diameter (Figure 5.10, Plate 5.6).

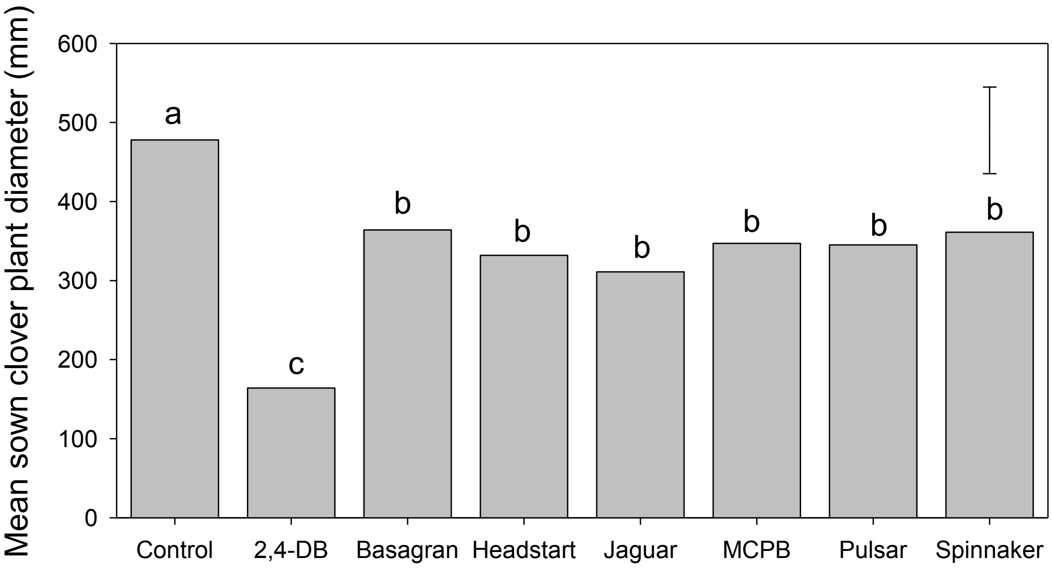

Figure 5.10 Mean diameter of sown clover plants for all treatments in Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand at 23 September 2016. Error bar is LSD for main effect of herbicide. Bars with a letter in common are not significantly different at α=0.05.

There was a cultivar*herbicide interaction (P=0.005) for the number of runners per plant in November, after grazing and recovery (Figure 5.11).

Unsprayed 'Antas' had about eight runners per plant (Plate 5.5), and bentazone, flumetsulam, and MCPB were not different to this. Remaining treatments had fewer (P=0.005) runners per plant with 4-5.

Unsprayed unweeded 'Denmark' had about three runners per plant, and 2,4-DB, bentazone, bromoxynil + diflufenican, and MCPB were not different to the control. flumetsulam, bentazone + MCPB, and imazethapyr increased the number of runners per 'Denmark' plant to 6-7.

Unsprayed ‘Narrikup’ also had about eight runners per plant, and only bromoxynil + diflufenican and 2,4-DB had fewer, with four and five runners respectively.

Runner numbers of 2,4-DB and MCPB treated ‘Woogenellup’ were reduced (P=0.005), with four and five runners respectively, compared to the unsprayed unweeded controls and all other plants at eight runners per plant (Figure 5.11).

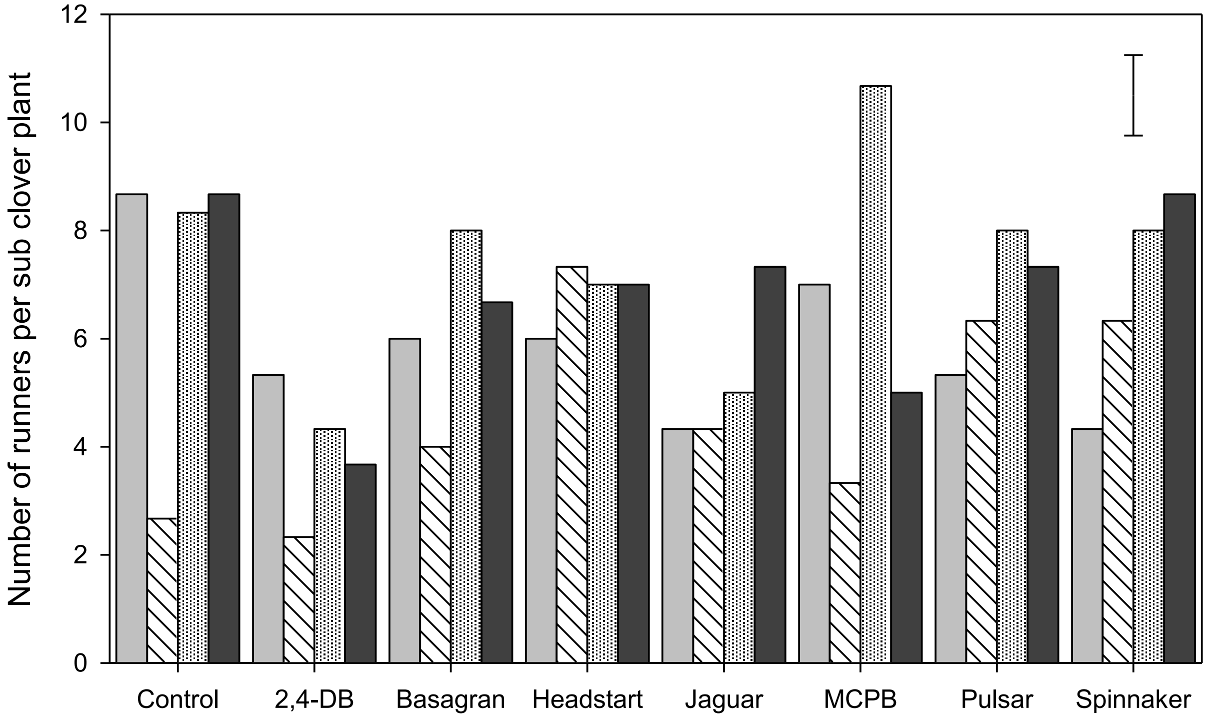

Figure 5.11 Number of runners per plant on 13 November 2016 for ‘Antas’ ( ) ‘Denmark’ ( ), ‘Narrikup’ ( ), ‘Woogenellup’ ( ), for all treatments in Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand. Error bar is LSD for cultivar*herbicide interaction.

By November there was a cultivar*herbicide interaction (P=0.004) for the number of reproductive nodes per plant. Most plots were reproductive and the number of potential reproductive nodes per plant was highest in flumetsulam treated ‘Narrikup’, unsprayed unweeded ‘Antas’, and MCPB treated ‘Narrikup’, with over 150 reproductive nodes per plant (Figure 5.12).

Flumetsulam treated ‘Narrikup’ had 237 reproductive nodes per plant, which was more (P=0.004) than the unsprayed unweeded control with 106 reproductive nodes per plant. 2,4-DB treated ‘Narrikup had 22 nodes per plant, which was reduced (P=0.004) compared to the control. All remaining herbicide treatments were not different to the unsprayed unweeded control.

Herbicide treatments of 'Antas' had 30-110 reproductive nodes, which was fewer (P=0.004) reproductive nodes than the unsprayed unweeded control of about 200.

'Woogenellup' plants, sprayed with 2,4-DB and MCPB had ~ 20 reproductive nodes per plant, which was fewer (P=0.004) than the 108 reproductive nodes per plant in the control. All remaining treatments were not different to the control.

Herbicide treated 'Denmark' plants showed no difference in number of reproductive nodes compared with the unsprayed unweeded control with only 15 reproductive nodes per plant (Figure 5.12).

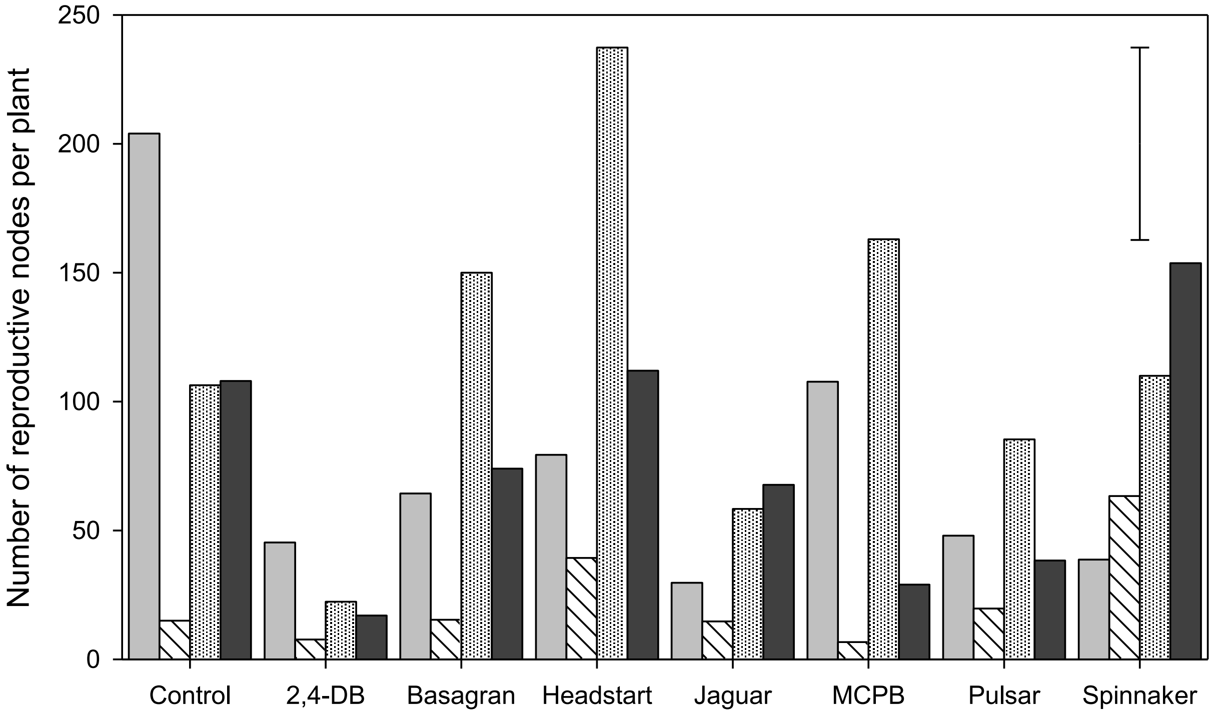

Figure 5.12 Number of reproductive nodes per plant on 13 November 2016 for 'Antas' ( ) 'Denmark' ( ), 'Narrikup' ( ), 'Woogenellup' ( ), for all treatments in Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand. Error bar is LSD for cultivar*herbicide interaction.

There was a cultivar* herbicide interaction (P=0.006) for the number of stage 3 burrs per plant. Flumetsulam treated 'Narrikup' had 71 stage 3 burrs per plant, which was greater than all other cultivars and treatments (Figure 5.13). There were three stage 3 burrs per plant for 2,4-DB treated 'Narrikup, which was reduced compared to the 41 in the control. All remaining treatments were not different to the unsprayed unweeded control.

Stage 3 burrs per plant for MCPB treated 'Antas' were the same as the unsprayed unweeded control, with 17-32. All remaining treatments had <11, and were reduced (P=0.006) compared with the control.

Stage 3 burr numbers per plant were the same for all treatments of 'Denmark', with 0-20, and not different to the unsprayed unweeded control with 7.

Stage 3 burr numbers of 'Woogenellup' were reduced (P=0.006) to <11 per plant by 2,4-DB, MCPB, bentazone, and bentazone + MCPB treatments, compared to 37 per plant in the unsprayed unweeded control. All remaining treatments were not different to the control, with 17-37 stage 3 burrs per plant.

All 2,4-DB treatments had no stage 3 burrs per plant, except 'Narrikup', which had two stage 3 burrs per plant (Figure 5.13). A comparison of the 2,4-DB effect on resident 'Woogenellup' plant growth and development can be seen in Plate 5.7 andPlate 5.8.

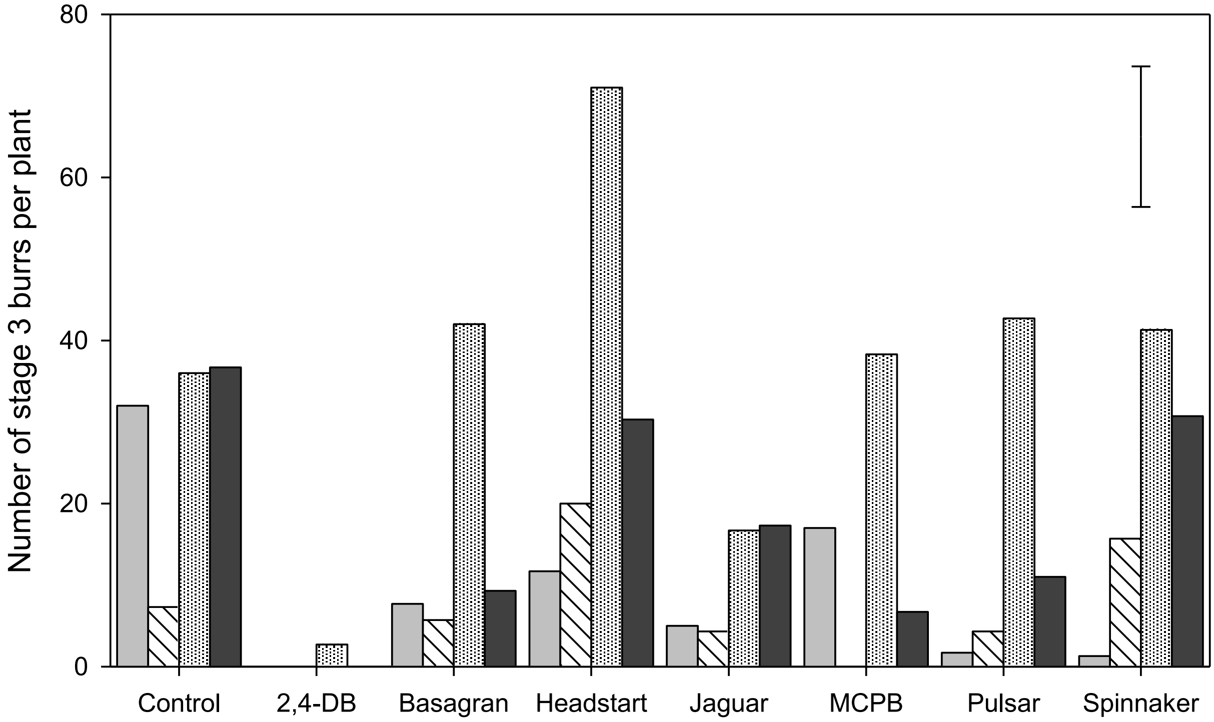

Figure 5.13 Number of stage 3 burrs per plant on 13 November 2016 for 'Antas' (■) 'Denmark' (▧), 'Narrikup' (▦), 'Woogenellup' (■), for all treatments in Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand. Error bar is LSD for cultivar*herbicide interaction.

Plate 5.5 Untreated unweeded control ‘Antas’ plants on 13 November 2016 from Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand.

Plate 5.6 Imazethapyr treated ‘Antas’ plants on 13 November 2016 from Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand.

Plate 5.7 Representative 2,4-DB treated 'Woogenellup' subterranean clover plant on 13 November 2016 from Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand. Supernumerary leaflet growth denoted by red arrow.

Plate 5.8 Unsprayed 'Woogenellup' subterranean clover plant on 13 November 2016 from Experiment 3 sprayed at the 1-2 trifoliate leaf stage on 14 June 2016, at Ashley Dene, Springston, Canterbury, New Zealand.

5.4 Discussion

The herbicide application at the 1-2 trifoliate leaf stage showed a difference in response among the sown subterranean clover cultivars. As a result, each herbicide should only be safely recommended on a cultivar basis after evaluation for phytotoxicity. However, the relatively small size of the New Zealand subterranean clover market means product registration and recommendations are unlikely. This may result in farmers using products off label and thus variable results can be expected. This discussion interprets these differential results to add to the body of knowledge but cannot be seen as an endorsement of particular treatments.

5.4.1 Seedling establishment

Seedling establishment was not different between cultivars, at 136 ± 10 plants per m^2. This indicated an ~60% field germination (Table 3.1) rate for all subterranean clover cultivars, and a ~30% germination rate for white clover. With relatively uniform emergence at the time of herbicide application, all responses for this experiment can be attributed to differences in response of the cultivars to the herbicides, weed competition, and environmental conditions.

5.4.2 Total yield potential

From this experiment, the total yield potential for this site in this season can be assessed by the total dry matter yield of the unsprayed unweeded control, which produced 4150 kg DM/ha from 22 March 2016 until 10 November 2016 (Figure 5.8). In most cases the total yield was reduced by the herbicide treatments, with only MCPB and bentazone treatments producing total yields for the season the same as the control. Total dry matter yields were reduced for all treatments in the September harvest, but after grazing and a month of regrowth, they had recovered, and by the November harvest all treatments had total dry matter yields the same as the unsprayed unweeded controls. Had rainfall occurred earlier in autumn, then differences in total dry matter yields may have been reduced even more, as grazing could begin earlier than September.

Of the mean 4150 kg/ha yield in the unsprayed unweeded control pastures, almost 50% was broadleaf weeds at 2000 kg DM/ha, 1000 kg/ha was resident 'Woogenellup' clover, 650 kg/ha grasses, and 150 kg/ha dead matter (Figure 5.8). Sown clover yields ranged from 200–26000 kg/ha depending on the cultivar and herbicide applied (Figure 5.7).

5.4.3 Glyphosate

Glyphosate was completely phytotoxic to clover, broadleaves, and grass weeds, with an EWRS score of 9.0 which meant the ground was completely bare prior to the first harvest in September. All physical yields were assumed to be 0 kg DM/ha, and eliminated from yield analyses. With this result, glyphosate applied at this rate would not be a recommended option for broadleaf weed control within subterranean clover containing pastures. While glyphosate is recommended safe on white clover at rates of 0.6 and 0.8 L/ha in Australia, and slightly susceptible at the applied rate of 1.0 L/ha (Riffkin *et al.* 2005) the white clover failed to persist at all after treatment at the higher 1 L/ha rate applied to ensure effective weed control, likely due to the low rainfall and therefore water stress the plant was under at the time of application.

5.4.4 2,4-DB

Total dry matter yields for 2,4-DB treated plots were reduced by 20%, with no increases in subterranean clover to compensate for this yield depression. Based on the combined dry matter and development results of this experiment, 2,4-DB at the applied rate was not suitable for establishing subterranean clover containing pastures and cannot be recommended. 2,4-DB treatments had the most visible phytotoxicity damage in surviving treatments, with a score of 4.8, higher than the unsprayed unweeded control, and all other cultivars. This suggests that 2,4-DB treated plots would have the greatest yield reductions relative to their unsprayed unweeded control. Only 'Antas' showed a visible reduction in sown clover dry matter yields compared to the unsprayed unweeded control, while all other cultivars were not different (Figure 5.7), showing 2,4-DB treatment provided no yield advantage for subterranean clover cultivars.

Despite minimal change in dry matter yields at the first harvest in September, 2,4-DB treated subterranean clover was reduced in size compared to the unsprayed unweeded controls (Table 5.3), and showed delayed development by at least one runner stage (Figure 5.9). This size reduction and developmental delay were still present in the November harvest, with the treated plants having fewer runners, reproductive nodes and stage 3 burrs per plant than the unsprayed unweeded controls (Plate 5.8, and Figure 5.10, Figure 5.11, Figure 5.12, and Figure 5.13). This reproductive delay is a cause for concern for the annual life cycle of subterranean clover. Its reproductive development is essential to ensure continued success in the pasture, particularly in the first year when seed set is often the priority. If plants are producing fewer seed carrying burrs (stage 3 burrs), and are doing so at a slower rate than normal, this could compromise the productivity and persistence of the pasture for future years. 2,4-DB is a synthetic auxin herbicide, where auxin is a plant growth hormone. When auxin levels are too high, natural growth is disrupted, mature cells grow abnormally, and new cells are prevented from forming. Mis-expression of *YUC1* in Arabidopsis embryo sacs has been found to act as a morphogenetic switch in expression between gametic and non-gametic cells, preventing full reproductive development (Zhao 2010), and a similar process is likely occurring in the 2,4-DB treated subterranean clover. This genetic/molecular mechanism is supported by Thimann (1939), who observed that auxin increases in plants can prevent the formation of lateral buds, and promote adventitious growth in already developed (mature) buds and axils, and the cost of further plant development. Further to this, although auxin inhibition (through overdose) is reversible, when prolonged over a period of some months, buds will slowly lose their ability to grow, and respond to auxin in any form.

Coupled with this developmental delay, the 2,4-DB only reduced broadleaf weeds by 65% compared to unsprayed unweeded controls, and this was one of the smallest reductions. Based on the combined sown and resident clover yields, subterranean clover was as disadvantaged by the 2,4-DB treatment as it was by the weed ingress in the controls. The limiting effect of 2,4-DB on plant size prevented subterranean clover plants from taking advantage of the space provided by the elimination of weeds, and space created was occupied by annual weed grasses (Figure 5.8). This makes 2,4-DB an ineffective herbicide for the purpose of increasing the subterranean clover content of a pasture.

5.4.5 MCPB

MCPB treatments had the smallest reduction in broadleaf weeds of all the herbicides applied, with only 55% broadleaf weeds controlled after herbicide application (Figure 5.8). There were no changes in dry matter yields for MCPB treated plots compared to the unsprayed unweeded controls, however there was no increase in clover content as a result of application (Figure 5.7). An average EWRS score of 3.4 for MCPB treated plots suggests slight phytotoxicity damage to the subterranean clover cultivars, and yield increases compared to unsprayed unweeded controls were not expected. Sown clover yields for all cultivars were not different to their unsprayed unweeded controls, and combined sown + resident clover yields were also the same (Figure 5.7). This suggests MCPB treated subterranean clovers were unable to take advantage of the space provided to them by broadleaf weed reduction. Annual weed grasses took advantage of the gaps, as in the 2,4-DB treatments, bringing the total dry matter for MCPB treatments for the season up to yield the same as the unsprayed unweeded control (Figure 5.8).

MCPB effects on development showed few early growth differences, with only ‘Narrikup’ showing a reduction in diameter compared to its unsprayed unweeded control (Table 5.3). This had no effect on the number of nodes per runner however, and all treated subterranean clovers had ~ 4 nodes per runner and were the same as the unsprayed unweeded control (Figure 5.9). By November, when plants had progressed to late reproductive development, all plants were reduced in size compared to their unsprayed controls (Figure 5.10) and development responses differed. Sown ‘Antas’ and resident ‘Woogenellup’ subterranean clovers showed a reduction in the number of reproductive nodes and stage 3 burrs per plant, and ‘Woogenellup’ also had a reduction in the number of runners per plant (Figure 5.11). ‘Narrikup’ had no difference compared to its unsprayed unweeded controls (Figure 5.12, andFigure 5.13). Based on these results, despite MCPB treatment not negatively affecting the subterranean clover dry matter yields, there was a reduction in development rates for ‘Antas’ and

‘Woogenellup’, and no subterranean clover cultivars showed a positive response to the MCPB treatment. This suggests it was also ineffective at the applied rate during establishment of subterranean clover in these pastures.

5.4.6 Bentazone

Bentazone was the only treatment with an EWRS score which showed no visible difference to the unsprayed unweeded control. This suggests that yields from this herbicide should be higher than, or no different to the unsprayed unweeded controls. Sown clover yields for all bentazone treated cultivars were not different to their unweeded unsprayed controls for both harvest dates (Figure 5.3, and Figure 5.5). However combined sown + resident clover yields were on average 650 kg/ha greater than the unsprayed unweeded controls. Furthermore, total dry matter yields for the season were the same as the unsprayed unweeded control (Figure 5.8). With a 70% reduction in broadleaf weeds, weed control was not as effective as some of the other treatments, but the increased combined clover yields suggest less phytotoxicity to subterranean clover. This was especially important in the case of ‘Antas’ which showed increased susceptibility yield depressions to most herbicides applied (Figure 5.7). However this reduced phytotoxic effect could have been due slower rates of photosynthesis preventing adverse effects (Melis 1999).

Bentazone is normally recommended for application in temperatures higher than 10 °C, with soil temperatures higher than 5 °C (Section2.5.2.1). In mid-June, average temperatures were 10°, just on the edge of the recommended temperature and decreasing. As a consequence, the efficacy would not be as expected compared to application in optimal conditions. With an earlier start to the growing season, treatments would be applied in warmer weather, and weed control may increase. As a photosystems II herbicide (Section 2.5.2), bentazone relies on plant metabolism for uptake of the herbicide and initiation of photodamage to susceptible plants. If plants are photosynthesising at a reduced rate, susceptibility can decrease, leading to reductions in weed control. Subterranean clover phytotoxicity damage from bentazone treatment may also be reduced if treatment is applied in warmer conditions. This is because the cooler temperatures increase the time the plant is exposed to the herbicide before it can degrade it, while in warmer temperatures, the active ingredient is rapidly assimilated and degraded by tolerant plants.

Early development was not affected by bentazone (Figure 5.9), but ‘Denmark’ plants were larger in diameter than their unsprayed unweeded controls, suggesting a benefit from weed removal. At the reproductive stage, ‘Antas’ showed a reduction in reproductive nodes (Figure 5.12) and stage 3 burrs, while ‘Woogenellup’ only showed a reduction in stage 3 burrs (Figure 5.13) compared to its unsprayed unweeded control. The impact on development was not as large as those caused by 2,4-DB and MCPB, as plants managed to produce ~10 stage 3 burrs per plant by the 13 November 2016. This result suggests that the effect was merely a delay in switch to development as a result of reduced growth rates while metabolising the active ingredient, and could recover in time.

Bentazone has potential as a herbicide for subterranean clover containing pastures, but the effect on plant development of 'Antas' and 'Woogenellup' remains a concern, and could impact the future of the pasture. Evaluation in a warmer application time should be assessed before any recommendations can be made.

5.4.7 Bentazone + MCPB

Bentazone + MCPB is a mixed formulation of MCPB (Section 5.4.5) + bentazone (Section 2.5.2.1), and clover responses were expected to be similar to those found in their treatments.

Bentazone + MCPB treated clover had an average EWRS score of 3.0, suggesting the sown clover yields would be reduced, or not different compared to the unsprayed unweeded control. In the September harvest, all cultivars yielded the same as their unsprayed unweeded controls except 'Antas', which yielded lower (Figure 5.4). As was discussed in Sections 5.4.5 and **Error! Reference source not found.**, this response of 'Antas' could be a result of higher uptake due to larger cotyledon and 1-2 trifoliate leaf size, metabolic rates and slow growth through cooler temperatures, or a lack of adaptation to the environmental conditions. By November, sown clover yields were not different to the unsprayed unweeded controls for all cultivars and the pasture recovered from bentazone + MCPB treatment, with total dry matter yields showed same as the unsprayed unweeded controls (Figure 5.6). Combined sown + resident clover yields for the season were not different to the unsprayed unweeded control, but total dry matter yields were reduced by 30% as a result of the 75% reduction in broadleaf weeds to 500 kg/ha, and the subterranean clover and annual grasses did not successfully fill the gaps produced by the herbicide as they had in others.

Bentazone + MCPB treated 'Denmark', was eight times larger than the unsprayed unweeded control in diameter, showing that while there may not have been a yield increase, 'Denmark began to take advantage of the space provided by broadleaf weed elimination. If the environmental conditions had been more favourable and germination had occurred earlier, this increase in plant diameter would likely have led to an increase in productivity and yields for 'Denmark'. This response was also seen in bentazone, whereas in MCPB treatments, 'Denmark' was not different to its unsprayed unweeded control (Table 5.3). By November, all subterranean clover mean plant diameters were reduced compared to the controls, but were not different to the MCPB or bentazone treatments. This change in plant diameter is most likely to herbicide treatment, and probably not increased clover plant density causing crowding due to the yield depressions observed. However, the smaller plant diameters could also be a response to grazing pressure. ‘Antas’ had fewer, while ‘Denmark’ had more runners per plant compared to plants in their unsprayed unweeded controls (Figure 5.11).

The number of reproductive nodes and stage 3 burrs per plant for ‘Antas’ followed the same trend as bentazone treatment, with a reduction. However MCPB treated ‘Antas had an increase in the number of stage 3 burrs per plant (Figure 5.12, and Figure 5.13). ‘Woogenellup’ also had a reduction in stage 3 burrs, which was seen in both the bentazone and MCPB treatments.

This varied response to the mixed bentazone + MCPB formulation of bentazone + MCPB shows that responses of subterranean clover cultivars cannot be predicted from responses to the individual chemicals. While Basagran was one of the highest yielding treatments for mean combined clover (Figure 5.8), the effect of bentazone + MCPB on yield was more similar to MCPB, and combined clover yields for the season were not different to the unsprayed unweeded control. There was also an unpredictable, long term plant development response, with reductions in plant size, and for ‘Antas’ and ‘Woogenellup’, also a potential decrease in seed set, which would not be compensated for through larger populations. In a better growing season, these effects may not have been as pronounced, but for early-winter applications, Pulsar cannot be recommended as an option in establishing subterranean clover-containing pastures as it confers no advantage. Despite high levels of weed control, there was no justifiable reason for its application, due to decreases in total dry matter yield, no change in subterranean clover yield, and unknown effects on long term persistence of the pastures, due to changes in reproductive performance. Investigation into the effects of bentazone + MCPB when applied in more optimal temperatures earlier in the year is advisable.

5.4.8 Bromoxynil + diflufenican

Bromoxynil + diflufenican treated sown clover, with an average EWRS score of 3.0 (Figure 5.2) indicated a reduction in yields, or no difference compared to the unsprayed unweeded controls. Early season yields of ‘Antas’ were reduced, while all other cultivars had no phytotoxicity effect on sown clover yields (Figure 5.3). However, resident clover yields were reduced by bromoxynil + diflufenican in the September harvest (Figure 5.4). After grazing and recovery, second harvest total dry matter yields were not different to the unsprayed unweeded controls (Figure 5.6), showing the ability of the pasture to recover from bromoxynil + diflufenican application. Bromoxynil + diflufenican treated ‘Narrikup’ yielded higher than its unsprayed unweeded control, while all other cultivars were the same as the unsprayed unweeded control (Figure 5.5). Broadleaf weeds for the season were reduced by 80%, while grass weeds were unaffected, leaving total season dry matter yields 20% lower than the unsprayed unweeded control (Figure 5.8).

Bromoxynil + diflufenican showed no visible reduction in early development compared to the unsprayed unweeded controls in September (Figure 5.9), and plant diameters of 'Denmark' and 'Monti' actually increased in response to the elimination of the broadleaf weeds (Table 5.3). However by November, mean plant diameters were reduced compared to unsprayed unweeded control plants (Figure 5.10), either as a response of herbicide treatment, grazing pressure, or crowding through increased clover plant density. 'Antas' and 'Narrikup' plants also had fewer runners per plant (Figure 5.11). The number of reproductive nodes per plant was also reduced in 'Antas' (Figure 5.12), and the number of stage 3 burrs per plant was reduced for all cultivars except 'Denmark' (Figure 5.13).

While bromoxynil + diflufenican provided increased weed control without damaging grasses, there was a developmental impact on all cultivars, seen in the reduction of stage 3 burrs. This could have an impact on the re-establishment of the pasture in the following year. There is potential for the use of bromoxynil + diflufenican in re-establishing pastures with high quality grass swards where weed control has become an issue. However the subterranean clover is more likely to establish well if not in direct competition with grasses. This is because, despite high levels of weed control, total combined clover yields were not higher than the unsprayed unweeded control yields. With total dry matter yields lower than the unsprayed unweeded control, and the long term effects of Jaguar on seed set not certain, bromoxynil + diflufenican cannot be recommended for use on establishing subterranean clover pastures at the 1-2 trifoliate leaf stage.

5.4.9 Flumetsulam

Flumetsulam appeared less phytotoxic to subterranean clover than most herbicide treatments. It provided a high level of long-lasting weed control, and allowed the sown and resident subterranean clover to take advantage of the gaps provided. Flumetsulam treatments had an average EWRS score of 2.6, just higher than the unsprayed unweeded control (Figure 5.2). This low EWRS meant at the September harvest, sown and resident clover yields were not different to the unsprayed unweeded controls, except for 'Antas' (Figure 5.3, and Figure 5.4). After grazing, sown clover yields of all cultivars recovered to be no different to the unsprayed unweeded controls (Figure 5.5). The combined sown + resident clover yields were 1.5 times higher for the season than the unweeded unsprayed control (Figure 5.8), and second harvest total dry matter yields were not different to the unsprayed unweeded controls (Figure 5.6). Broadleaf weeds were controlled by 95%, with <100 kg/ha remaining, while grasses were unaffected. These yields shows that flumetsulam had no adverse phytotoxic effect on yields of subterranean clover, high levels of weed control, and plants were able to move into the space previously occupied by weeds.

In conjunction with these results, flumetsulam had little effect on early plant growth, with no changes in nodes per runner (Figure 5.9), and there was a positive response for 'Denmark' with a larger diameter than the unsprayed unweed control plants (Table 5.3). However by the November harvest, all cultivars were smaller in diameter than the unsprayed unweeded control plants (Figure 5.10), and 'Denmark' had more runners per plant while the other cultivars were not different to the controls (Figure 5.11). This decrease in plant size was probably not a phytotoxicity effect of the flumetsulam, but a physiological response to high population densities that forced plants to be compact, as combined sown + resident clover yields of flumetsulam treated clovers were 700 kg/ha higher than the unsprayed controls (Figure 5.8).

Flumetsulam appeared to have advanced the rate of development of 'Narrikup', which had more reproductive nodes and stage 3 burrs per plant than the unsprayed unweeded control. 'Antas' had a reduction in the number of reproductive nodes (Figure 5.12) as well as the number of stage 3 burrs compared to the unsprayed unweeded control (Figure 5.13). Based on these results, sown 'Narrikup' and 'Denmark', and the resident 'Woogenellup' subterranean clover cultivars were tolerant of flumetsulam treatment, while 'Antas' appeared susceptible with a phytotoxicity response of development delay, despite not having a difference in dry matter yields.

5.4.10 Imazethapyr

Imazethapyr was the most successful herbicide treatment. An average EWRS score of 3.0 indicated a potential decrease in yields of sown clover, and this occurred for 'Antas' in the September harvest. All other cultivars were not different to their unsprayed unweeded controls (Figure 5.3). Resident clover yields were also reduced in the September harvest compared to the controls, suggesting a phytotoxic response of 'Woogenellup' to imazethapyr. Despite this early vegetative response, post grazing the sown clovers recovered, with both 'Antas' and 'Narrikup' producing higher yields than their unsprayed unweeded controls (Figure 5.5). Combined sown + resident clover yields were 1000 kg/ha higher than the unsprayed unweeded control (Figure 5.6). Broadleaf weeds and grass weeds were each reduced to <50 kg/ha (Figure 5.8).

There was an early effect of imazethapyr on 'Narrikup' plant diameter, which was reduced compared to the unsprayed unweeded control (Table 5.3), but there was no effect on development in September (Figure 5.9). In November, plants were reduced in diameter compared to the unsprayed unweeded controls (Figure 5.10), and 'Antas' had fewer runners per plant, while 'Denmark' had more runners per plant (Figure 5.11). 'Antas' also had a reduction in the number of reproductive nodes per plant (Figure 5.12) and stage 3 burrs per plant (Figure 5.13). Reductions in plant size were probably again not due to treatment, but to the greater abundance of subterranean clover causing crowding, which reduced plant sizes. With mean combined clover yields higher than the controls, and recovery of all cultivars after grazing coupled with broadleaf and grass weed control of more than 95% for over six months imazethapyr is a promising herbicide for broadleaf weed control when subterranean clover. This is because the subterranean clover was most able to recover from the effect of application and take advantage of the increase in space and lack of competition afforded by the high level of broadleaf and grass weed control.

5.4.11 White clover

'Kopu II' white clover consistently yielded 1000 kg/ha less than the sown subterranean clover in this experiment (Figure 5.7). When combined with the resident clover, yields were 2000 kg/ha lower (Figure 5.8). This was due to the lower than average rainfall, and the lack of adaptation of white clover to dry environments, especially when establishing. This poor performance highlights the need for inclusion of subterranean clover in areas which have variable seasonal rainfall. White clover showed no difference in response to any of the herbicide treatments, consistently yielding 300 ± 200 kg/ha for the season in all herbicide treatments with no effects on plant diameter in the September harvest (Table 5.3). This confirms that none of these herbicides except glyphosate affected white clover, and they are all currently recommended for it (Novachem 2016).

5.4.12 Subterranean clover

Sown subterranean clover yields for treatments applied at the 1-2 trifoliate leaf stage suggest that 'Narrikup' was most tolerant of the widest range of herbicide treatments. Those that provided higher levels of weed control allowed it to be more productive and take advantage of the gaps created. This was seen in the imazethapyr and flumetsulam treatments, especially for 'Narrikup' treated with imazethapyr where yields were 2.6 times higher than the unsprayed unweeded control (Figure 5.7). Despite lower sown clover yields in the imazethapyr and flumetsulam treated 'Antas', 'Denmark' and 'Monti' plots, there was no difference in total yields when sown subterranean clover and resident 'Woogenellup' subterranean clover were combined (Figure 5.8). This shows that subterranean clover was capable of taking advantage of the gaps created by weed control at the seedling stage, and their ability to do so in this experiment was affected by the environmental conditions. All treatments recovered to have total dry matter yields not different to the unsprayed unweeded control by the November harvest. Those that arrested weed growth early on, and allowed subterranean clover to dominate produced more legume.

'Denmark' consistently had fewer reproductive nodes and stage 3 burrs than the other subterranean clover cultivars, but this was expected as it is a later flowering, smaller leafed cultivar. The only treatment which affected development of 'Denmark' was 2,4-DB, which prevented stage 3 burrs from occurring in November. With earlier autumn rainfall, 'Denmark' would probably have produced more, as it has a longer growing season requirement than 'Narrikup' and 'Woogenellup' (Lucas *et al.* 2015).

5.4.13 Environmental effects

Due to the lack of autumn rainfall (Figure 5.1), experiments were not sown until late March, after adequate rain had occurred to soften the dry ground enough for the drill to penetrate. After sowing, a lack of rainfall delayed emergence until 20 May when 132 mm fell. This finally allowed germination and seedling emergence. As a result of this delay to the start of the growing season, plants emerged into an environment limited by temperature, and as such, we would expect to see reduced yields for the season (Moot *et al.* 2003) as a consequence of longer times to fulfil the thermal time requirement for leaf appearance (phyllochron) and therefore had reduced light interception. This was seen in the lower than expected yields in 'Antas', 'Denmark' and 'Monti'.

'Antas' is a subspecies *brachycalycinum* cultivar, and another *brachycalycinum* cultivar, 'Clare' has shown variable herbicide tolerance and phytotoxicity responses in different years, due to differences in rainfall in growing seasons (Evers *et al.* 1993). Due to the persistence shown in this season, it is likely that 'Antas' could perform better after herbicide treatment in more ideal growing seasons, with increased rainfall. Further investigation into the effects of herbicides on its seed set and subsequent regeneration may be warranted.

5.5 Conclusions

The higher / unchanged sown clover DM yields in imazethapyr and flumetsulam treatments, coupled with their >95% broadleaf weed control and grass weed suppression suggests that ALS inhibiting herbicides may most ensure success when establishing a new subterranean clover containing pasture. Bentazone is also a potential option, and as a photosystems II inhibitor, however with its temperature limitations, it is not always suitable, and its performance from spraying this late in autumn at 10 °C was unexpected.

'Denmark' responded to elimination of weeds with increases in diameter, showing potential for increased productivity and yields. These could be observed in a season with earlier germination. 'Antas' appeared susceptible to changes in development as a result of herbicide application, again, this could just be a delay as a result of the shortened growing season, and an earlier germination may present less pronounced delays.

2,4-DB is not suitable for use on subterranean clover pastures containing as a result of its detrimental effect on the rates of plant reproductive development of all five evaluated subterranean clover cultivars.

6 EXPERIMENT 4: HERBICIDE APPLICATION AT THE 4+ TRIFOLIATE LEAF STAGE

6.1 Introduction

With the temperate climate and shortened subterranean clover growing season in New Zealand, application of herbicides is often deferred until early winter to prevent plant loss through herbicide damage. However, the potential effects of weed ingress on establishment when this is done can be high (Frame & Newbould 1986) as weeds will always establish earlier in the season, often outcompeting the smaller clover plants when conditions finally allow them to emerge. If this occurs, even with appropriate management it is difficult to generate a high enough seed set to ensure future regeneration (Smetham 2003).

This chapter fulfils Objective 3 (Figure 1.1), and reports the effects of herbicides on subterranean clover cultivars, as in Experiment 3 (Section 5.1), but when applied at the later 4-6 trifoliate leaf stage and reports on the difference between the application times.

6.2 Materials and methods

Materials and methods were as described in Chapter 5, Section 5.2

6.3 Results

6.3.1 Seedling establishment

Seedling number per m^2 was different among cultivars, but not different (P=0.114) between the Experiment 3 unsprayed unweeded controls 28 days after treatment application, and the unsprayed Experiment 4 plots. Seedling numbers were highest for 'Narrikup', 'Denmark' and 'Antas' at ~260 ± 38/m^2. This was higher (P=0.048) than 'Monti' and white clover with ~123 ± 38/m^2 (Table 6.1).

Table 6.1 Average seedling number per 1 m^2 for cultivars of Experiment 3 (1-2 leaf application) unsprayed unweeded controls and Experiment 4 (4-6 leaf application) for all subterranean clover cultivars and white clover prior to Experiment 4 herbicide application on 12 July 2016 at Ashley Dene, Springston, Canterbury, New Zealand.

	1-2 leaf application	4-6 leaf application	Cultivar mean
'Antas'	317	190	253
'Denmark'	289	238	263
'Monti'	113	127	120
'Narrikup'	259	272	266
White clover	127	126	126
P-value	0.114		0.048
e.s.e.	44		38

6.3.2 Phytotoxicity assessment

EWRS phytotoxicity scores of subterranean clover plants treated with herbicide at the 4+ trifoliate leaf stage increased (P<0.001) compared to unsprayed unweeded controls over time.

EWRS phytotoxicity scores of MCPB, 2,4-DB, and bentazone were 2.0-2.5 by 7 DAA, and higher (P<0.001) than their controls. All other treatments were not different to their controls.

By 28 DAA, the EWRS phytotoxicity score of glyphosate was 7.7, and exceeded the commercially allowable threshold. EWRS scores for bromoxynil + diflufenican, MCPB, flumetsulam, and bentazone + MCPB were 2.5-3.3, all still within the commercial limit, but different (P<0.001) to their controls. 2,4-DB, imazethapyr, and bentazone were the same as their controls.

At 63 DAA, bentazone had recovered to an ERWS score the same as its control. Bentazone + MCPB, bromoxynil + diflufenican, flumetsulam, and imazethapyr were tightly clustered together, with scores from 2.9-3.2. MCPB was higher than these, and only just within commercial limits with an EWRS score of 4.7, and 2,4 DB was higher (P<0.001) again, with a score of 5.8, just over the commercially acceptable threshold (Figure 6.1). Glyphosate treated plots had an EWRS score of 9.0, all plants were dead and plots were bare.

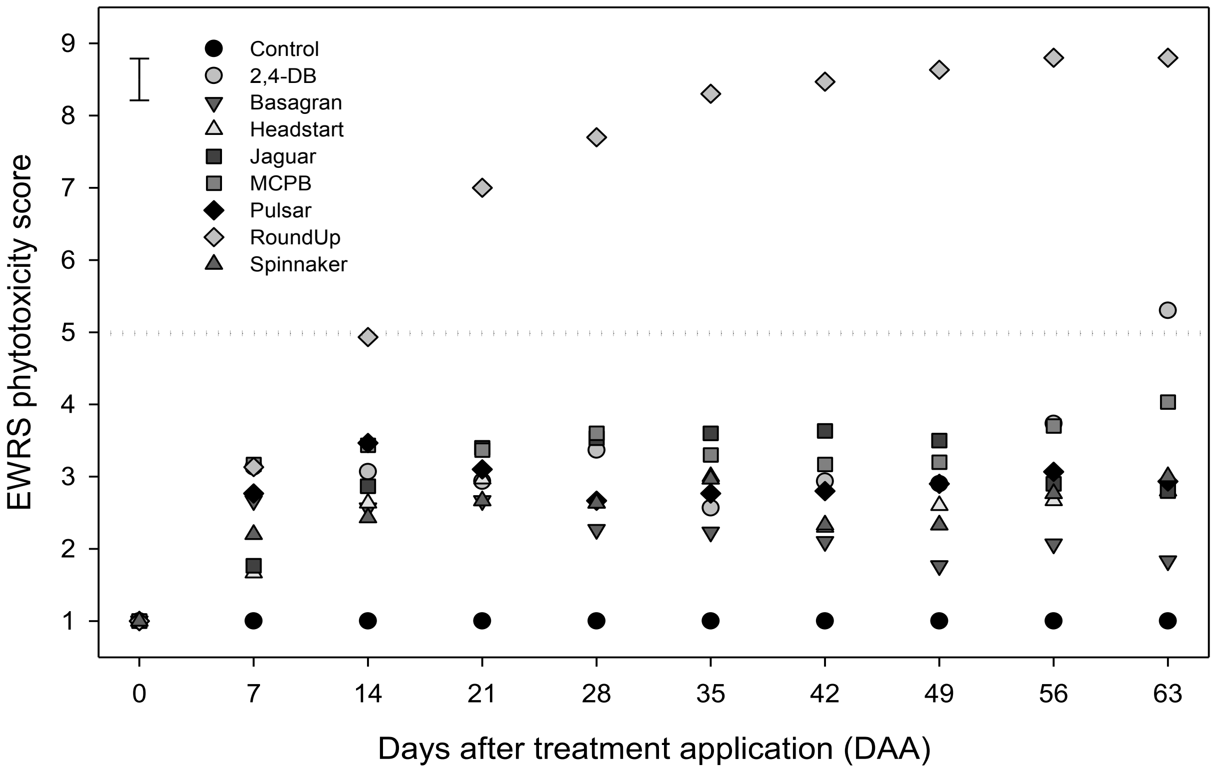

Figure 6.1 Average EWRS phytotoxicity scores for all treatments in Experiment 4 for 63 days after herbicide application (DAA) from 10 July 2016 at C9BS Ashley Dene Farm, Springston, Canterbury, New Zealand after herbicide application on the 14 June 2017 at the 4-6 trifoliate leaf stage. Error bar is LSD for treatment*time interaction.

6.3.3 Harvest 1: 20 September 2016

There was a herbicide*cultivar interaction for sown clover yields of plants treated at the 4+ trifoliate leaf stage. Sown clover yields of 575-700 kg DM/ha in unsprayed unweeded and bentazone treated ‘Narrikup’ were higher (P=0.002) than all other cultivars and treatments (Figure 6.2).

Sown clover yields for imazethapyr, bentazone + MCPB, flumetsulam, and MCPB treated ‘Narrikup’ at 280-400 kg DM/ha were lower (P=0.002) than controls, but higher (P=0.002) than 2,4-DB treated ‘Narrikup’ which was the lowest yielding with 120 kg DM/ha sown clover.

Sown clover yields of bentazone, flumetsulam and 2,4-DB treated ‘Antas’ were the same as the control, with 200-330 kg DM/ha. All remaining treatments yielded less (P=0.002) sown clover than the control, with <170 kg DM/ha.

Sown clover yields of treated ‘Denmark’ and ‘Monti’ and were not different to each other or their controls at 60-80 kg DM/ha. White clover was not different to the unsprayed unweeded control at 20 kg DM/ha for any herbicide application (Figure 6.2).

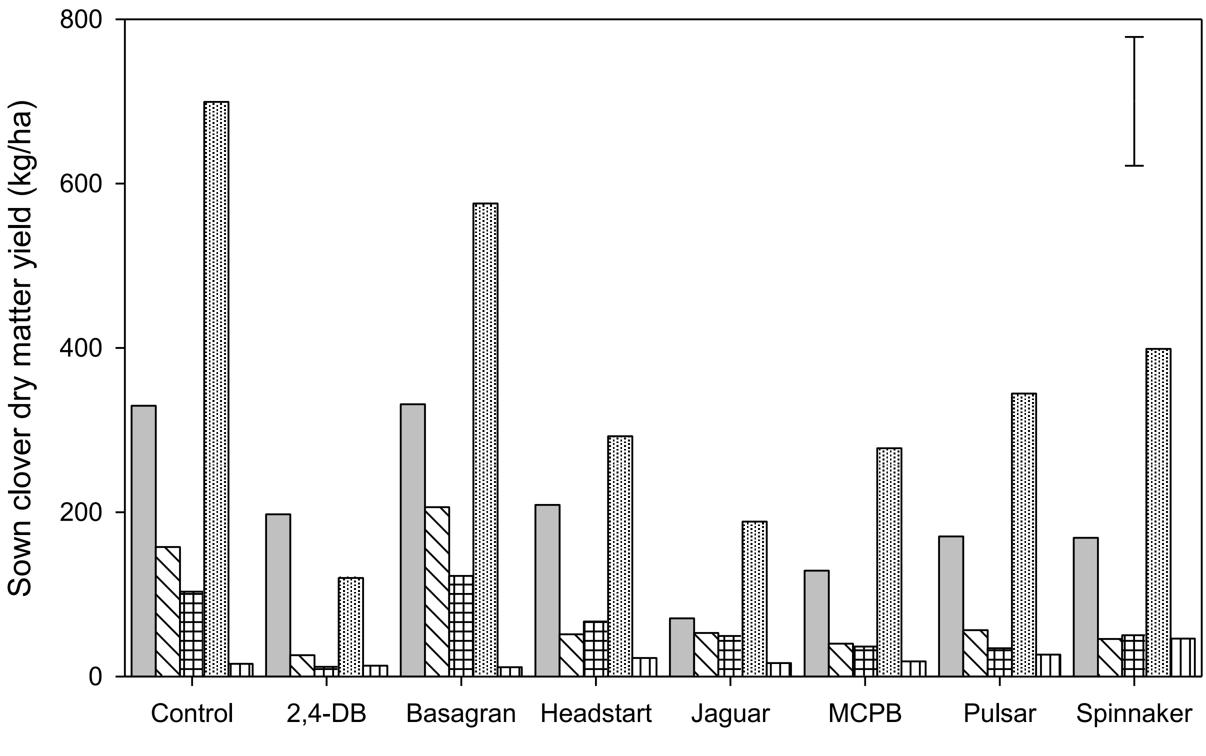

Figure 6.2 Sown clover dry matter yields of ‘Antas’ (■) ‘Denmark’ (▧), ‘Monti’ (▦), ‘Narrikup’ (▒), white clover (▥), for all herbicide treatments in Experiment 4, sprayed at the 4-6 trifoliate leaf stage on 12 July 2016, at Ashley Dene, Springston, Canterbury, New Zealand. Harvested 20 September 2016. Error bar is LSD for cultivar*herbicide interaction.

There was no evidence of a sown cultivar*herbicide interaction for yields of resident 'Woogenellup' clover (P=0.087), broadleaf weeds (P=0.169), grass weeds (P=0.346), or dead matter (P=0.364).

Resident 'Woogenellup' subterranean clover yields of 250-360 kg DM/ha were produced in bentazone, controls, and flumetsulam treatments. All other herbicide treatments yielded less (P=0.010) resident clover than the controls, with <140 kg DM/ha (Figure 6.3).

Broadleaf weed yields were highest in the unsprayed unweeded controls, with 1620 kg DM/ha, and these were greater (P<0.001) than all herbicide treatments. Broadleaf weed yields were lowest in imazethapyr and flumetsulam treatments, with <120 kg DM/ha (Figure 6.3).

Grass weed yields were highest in bromoxynil + diflufenican treatments, with 390 kg DM/ha, and these were greater (P=0.030) than grass weed yields of 120 kg DM/ha in the control. Grass weed yields for all remaining treatments yielded <280 kg DM/ha and were the same as the control.

Total dry matter yields were higher (P<0.001) in the unsprayed unweeded controls at 2380 kg MD/ha than all other herbicides with <1400 kg DM/ha (Figure 6.3).

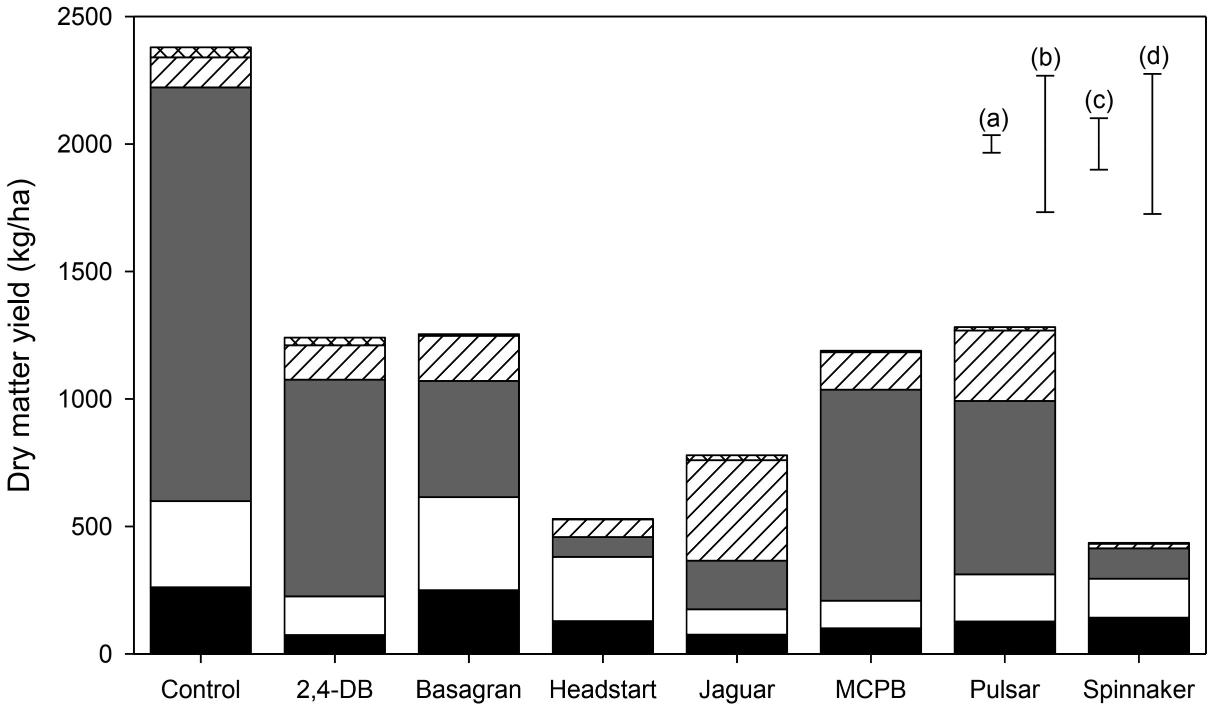

Figure 6.3 Mean dry matter yields and composition for herbicide treatments of Experiment 3, applied at the 4-6 leaf stage on 12 July 2016, harvested 20 September 2016 at Ashley Dene, Springston, Canterbury, New Zealand. Categories are sown clover (■), resident clover (□), broadleaf weed (■), grass weed (▨) and dead matter (⊠). Error bars are LSD for main effect of herbicide on (a) resident clover; (b) broadleafs; (c) grass weeds; (d) total dry matter.

6.3.4 Harvest 2: 11 November 2016

There was no cultivar*herbicide interaction for yields of sown clover (P=0.589), resident 'Woogenellup' clover (P=0.200), broadleaf weeds (P=0.084), grass weeds (P=0.316), or dead matter (P=0.072). There was a main effect. There was no main effect of herbicide on, sown clover (P=0.108) or on resident clover (P=0.389) grass weeds (P=0.188), dead matter (P=0.252) or total dry matter (P=0.147) yields.

There was a main effect of cultivar for sown clover yields. Sown clover yields were greatest (P=0.002) for 'Antas' and 'Narrikup', with 500 - 600 kg DM/ha, all other cultivars yielded lower.

There was a main effect of herbicide on combined (sown + resident) clover (P=0.030). Combined clover yields were higher than the unsprayed unweeded controls in imazethapyr and flumetsulam, with 1400-1800 kg DM/ha while all other herbicides were not different to the unsprayed unweeded control at 1000 kg DM/ha.

There was a main effect of herbicide on broadleaf weeds. Broadleaf weed yields were 970 kg DM/ha in unsprayed unweeded controls, higher (P<0.001) than all other herbicides. Broadleaf weed yields were lowest in bentazone, flumetsulam, and imazethapyr with 120-210 kg DM/ha (Figure 6.5). Remaining herbicides had yields of 370-560 kg DM/ha, with no differences.

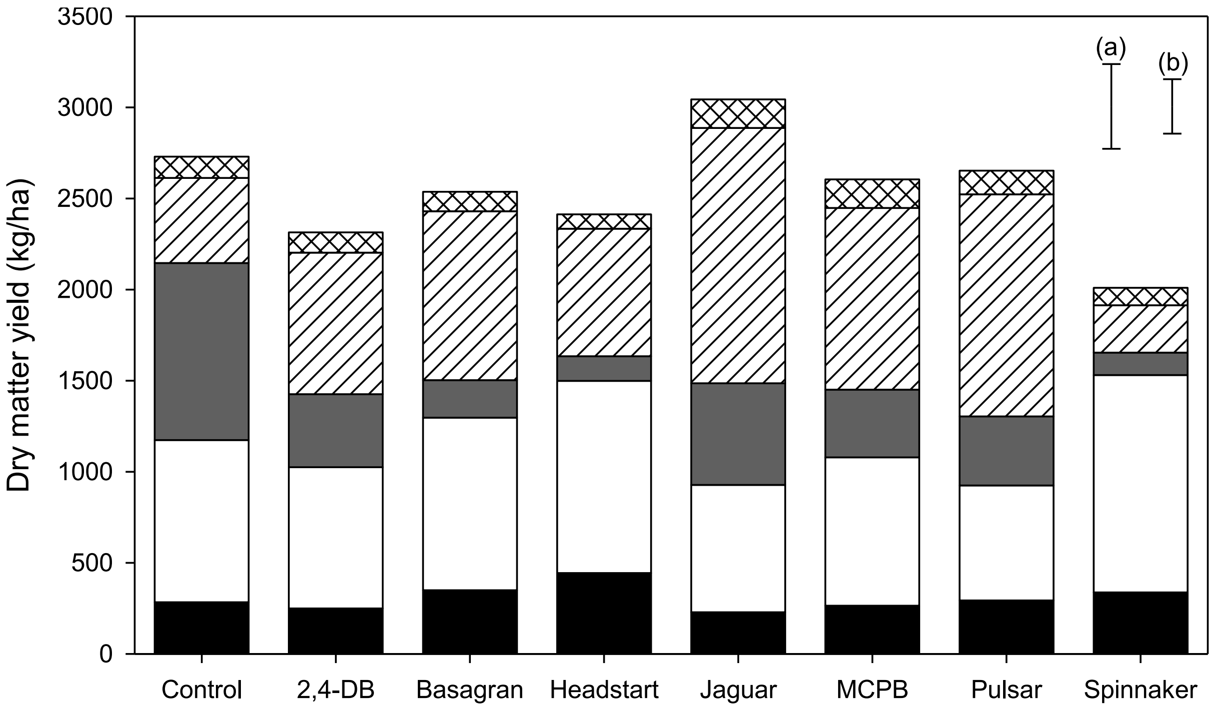

Figure 6.4 Mean dry matter yields and composition for herbicide treatments of Experiment 4, applied at the 4-6 trifoliate leaf stage on 12 July 2016, harvested 11 November 2016 at Ashley Dene, Springston, Canterbury, New Zealand. Categories are sown clover (■), resident clover (□), broadleaf weed (■), grass weed (▨) and dead matter (⊠).Error bars are LSD for main effect of herbicide on (a) combined (sown+resident) clover; (b) broadleaf weeds.

6.3.5 Total harvest yields

There was no cultivar*herbicide interaction for total season sown clover yields, but main effects for cultivar and herbicide.

Average sown clover yields of 830-855 kg DM/ha for 'Antas' and 'Narrikup' were higher (P<0.001) than all other cultivars, at <225 kg DM/ha.

Sown clover yields of 420-600 kg DM/ha for bentazone, flumetsulam, imazethapyr, and bentazone + MCPB were not different to the unsprayed control, which had 545 kg DM/ha. Sown clover yields of MCPB, 2,4-DB and bromoxynil + diflufenican were lower (P=0.006) than the unsprayed control, as well as bentazone and flumetsulam (Figure 6.5).

There was no evidence of a sown cultivar*herbicide interaction for yields of resident 'Woogenellup' clover (P=0.191), broadleaf weeds (P=0.270), grass weeds (P=0.659), or dead matter (P=0.058).

There was no main effect of herbicide for resident 'Woogenellup' clover (P=0.302) yields, at 1100 kg DM/ha. There was no main effect of herbicide for combined (sown + resident) (P=0.112) among herbicides, at 1550 kg DM/ha.

Average broadleaf weed yields were greater (P<0.001) in unsprayed unweeded controls, with 2595 kg DM/ha, than all other treatments. Broadleaf weed yields of bentazone, imazethapyr, and flumetsulam were <660 kg DM/ha, and imazethapyr and flumetsulam yields were lower (P<0.001) than all herbicides except bentazone. 2,4-DB had the highest weed yields of all herbicide treatments, with 1250 kg DM/ha, and this was not different to MCPB and bentazone + MCPB treatments, but greater than all others (Figure 6.5). Broadleaf weed yields of remaining herbicides were not different from one another.

Total dry matter yields were higher (P<0.001) in unsprayed unweeded controls at 5100 kg DM/ha than all herbicides. Weed density in this section of the paddock was higher than in Experiment 3, with 1000 kg DM/ha more broadleaf weeds. Flumetsulam and imazethapyr had the lowest (P<0.001) total dry matter yields with 2400-2900 kg DM/ha (Figure 6.5).

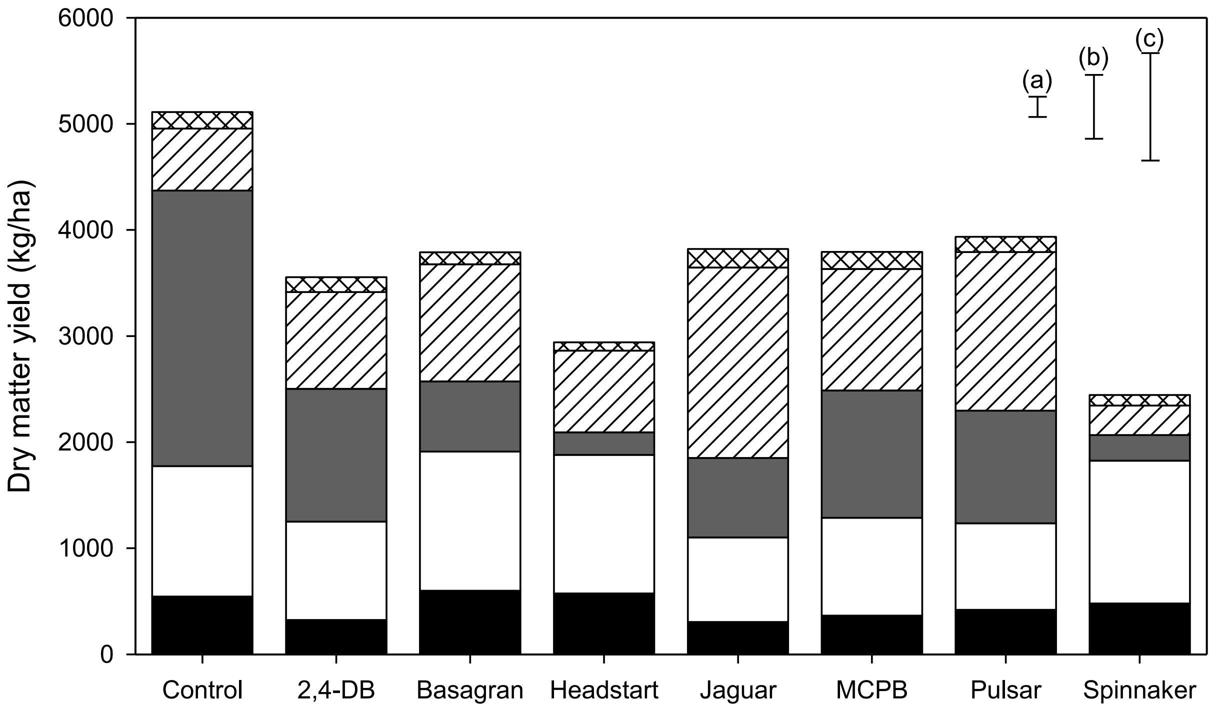

Figure 6.5 Total mean dry matter yields and composition for growth from March to 10 November 2016 for all herbicide treatments of Experiment 4, applied at the 4-6 trifoliate leaf stage on 12 July 2016 at Ashley Dene, Springston, Canterbury, New Zealand. Categories are sown clover (■), resident clover (□), broadleaf weed (■), grass weed (▨) and dead matter (⊠). (a) is LSD for herbicide effect on sown clover yield; (b) is LSD for herbicide effect on broadleaf weed yield; (c) is LSD for herbicide effect on total dry matter yield.

6.3.6 Plant development

6.3.6.1 September 2016:

There was a cultivar*herbicide interaction (P<0.001) for plant diameter in the September 2016 harvest (Table 6.2). ‘Antas’ unsprayed control plants measured 185 ± 35 mm in diameter. 2,4-DB treated plants were reduced (P<0.001) in size, at 67 ± 35 mm, and flumetsulam treated plants were larger (P<0.001) at 278 ± 35 mm.

Unsprayed ‘Denmark’ was the smallest plant, at 23 ± 35 mm, and only 2,4-DB, MCPB and imazethapyr caused no increase in plant diameter. Remaining herbicides were larger (P=0.001) than the unsprayed control, but not different to each other, at 132-184 cm in diameter.

‘Narrikup’ plants were the largest. Unsprayed ‘Narrikup’ was 262 ± 35 mm, only 2,4-DB and MCPB treated ‘Narrikup’ were smaller than the control at 147-152 mm.

Treated white clover plants were not different to the unsprayed control at 60 ± 35 mm except bromoxynil + diflufenican treated white clover plants which were larger (P<0.001) at 145 ± 35 mm (Table 6.2).

Table 6.2 Mean sown clover plant diameter on September 23 2016 for all treatments of subterranean clover in Experiment 4 sprayed at the 4-6 trifoliate leaf stage on 12 July 2016, at Ashley Dene, Springston, Canterbury, New Zealand. * indicates different to control for cultivar*herbicide interaction (P<0.001 e.s.e.=35).

Treatment	Antas	Denmark	Monti	Narrikup	White Clover
Control	185	23	52	262	60
2,4-DB	67 *	77	58	152 *	95
Basagran	173	169 *	72	240	46
Headstart	278 *	184 *	117	301	145 *
Jaguar	203	160 *	103	289	96
MCPB	240	80	131 *	147 *	93
Pulsar	216	132 *	127 *	295	49
Spinnaker	151	83	60	266	44

At the September harvest, There was no interaction between herbicides and cultivars only herbicide (P<0.001) and cultivar (P<0.001) main effects. All cultivars were at different (P<0.001) growth stages. 'Narrikup was the most advanced, with five nodes per runner. This was followed by 'Antas' with four nodes per runner, and then 'Denmark', which averaged 3.5 nodes per runner. 'Monti' had the lowest growth stage, with 2.5 nodes per runner.

Plant development was delayed (P<0.001) by 2,4-DB, at between two and three nodes per runner compared to all other treatments with about four nodes per runner (Figure 6.6). There was no difference in development stage among remaining herbicides and the unsprayed unweeded controls.

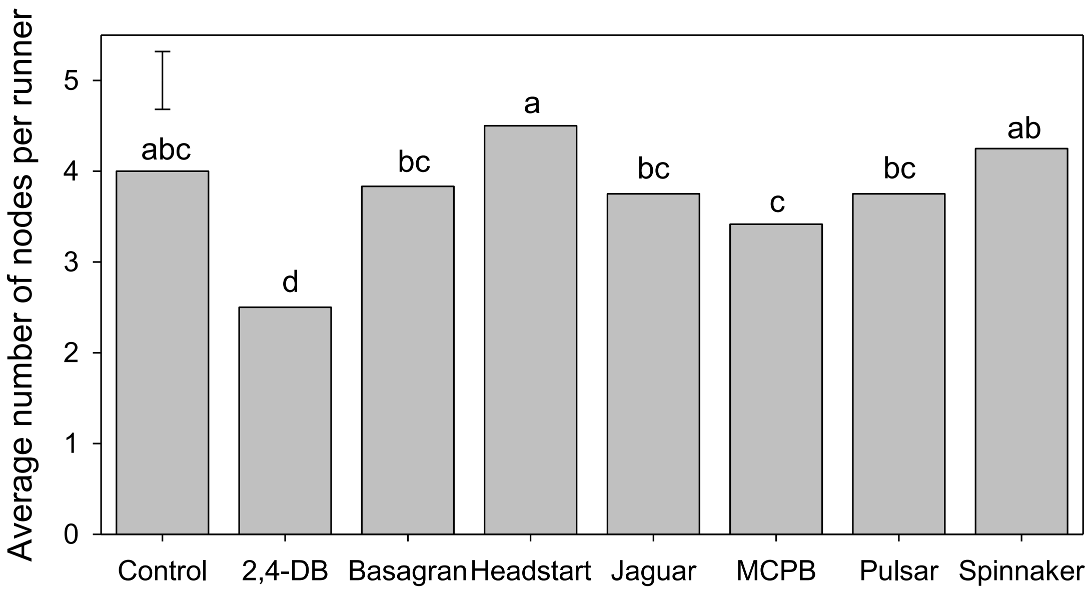

Figure 6.6 Mean number of nodes per runner for treatments of Experiment 4 at 23 September 2016, with treatments applied on 12 July 2016 at the 4-6 trifoliate leaf stage at Ashley Dene, Springston, Canterbury, New Zealand. Error bar is LSD for main effect of herbicide. Bars with a letter in common are not significantly different at α=0.05.

6.3.6.2 November 2016:

There was no cultivar*herbicide interaction for plant diameter after grazing and recovery at the November harvest, only primary effects of cultivar and treatments. ‘Narrikup’, ‘Woogenellup’, and ‘Antas’ at 365-384 mm in diameter were larger (P<0.001) than ‘Denmark’ at 163 ± 23 mm.

Unsprayed subterranean clover plants at a mean 394 mm diameter were not different to imazethapyr, bentazone + MCPB, bentazone, and flumetsulam treatments. 2,4-DB and bromoxynil + diflufenican treated plants were the smallest (P<0.001) at 200 and 290 mm, respectively. MCPB was smaller than the unsprayed control plants, but larger than 2,4-DB treated plants at 300 mm (Figure 6.7).

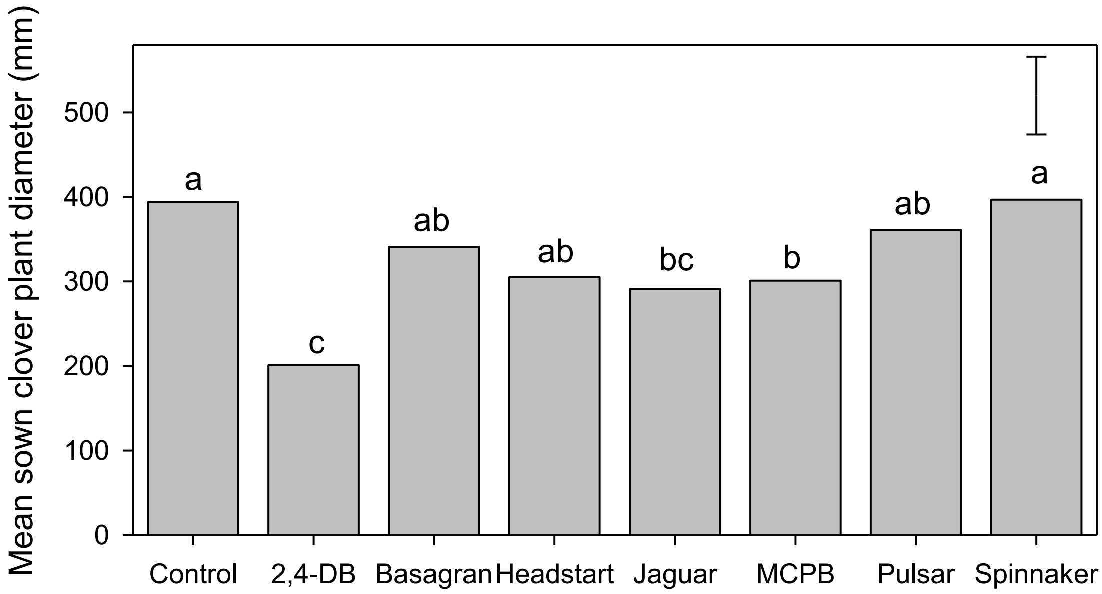

Figure 6.7 Mean diameter of sown clover plants for all treatments in Experiment 4 sprayed at the 4-6 trifoliate leaf stage on 12 July 2016, at Ashley Dene, Springston, Canterbury, New Zealand on 13 November 2016. Bar is LSD between treatments. Bars with a letter in common are not significantly different at α=0.05.

There was a herbicide*cultivar interaction (P<0.001) for the number of runners per plant for subterranean clover at the November 2016 harvest.

Unsprayed ‘Antas’ had ~7 runners per plant 2,4-DB, bentazone, MCPB and Headstart treated plants were not different, while bentazone + MCPB, imazethapyr, and bromoxynil + diflufenican treated plants had fewer (P<0.001) with ~4 (Figure 6.8).

With 5-7 runners per plant, bentazone, imazethapyr and flumetsulam treated Denmark had more (P<0.001) runners than the unsprayed ‘Denmark’ plants with ~2.

Unsprayed 'Narrikup' had the most runners, with ~8, and bentazone, flumetsulam, MCPB, bentazone + MCPB, and imazethapyr treated plants were not different. 2,4-DB and bromoxynil + diflufenican treated plants had fewer runners, with ~5 per plant (Figure 6.8).

Resident 'Woogenellup' plants were not affected by treatment, with 5-8 runners per plant (Figure 6.8).

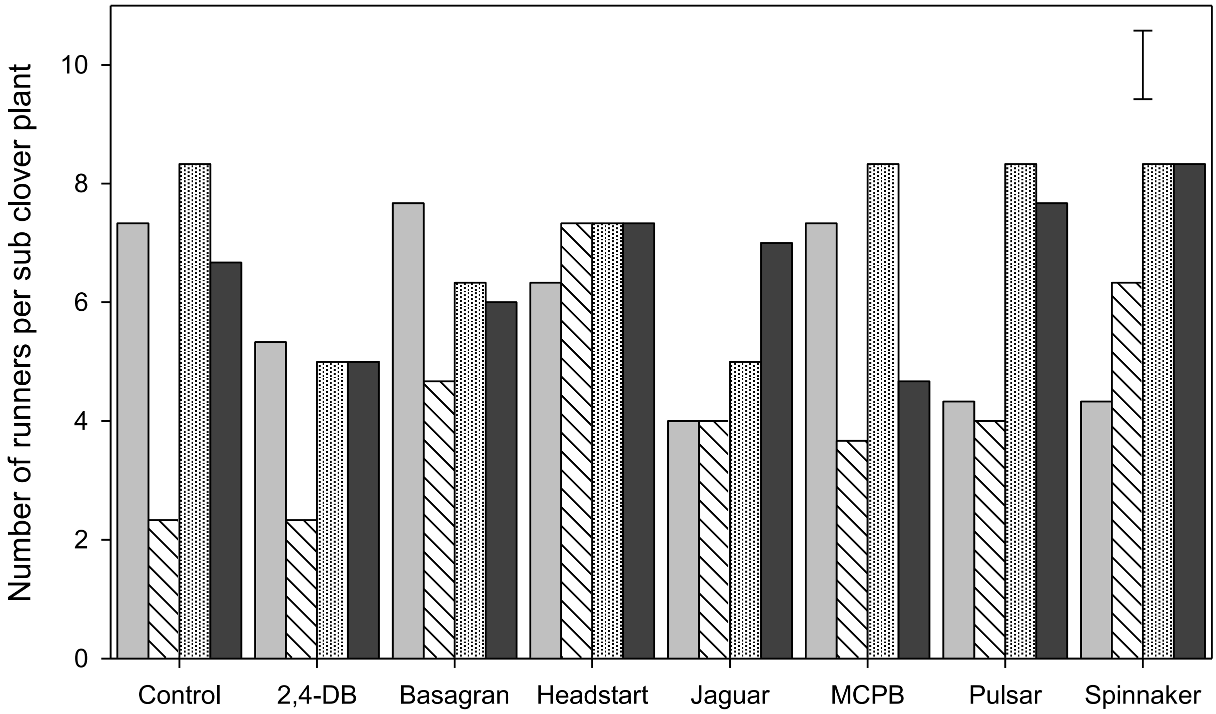

Figure 6.8 Number of runners per plant on 13 November 2016 for 'Antas' ( ) 'Denmark' ( ), 'Narrikup' ( ), 'Woogenellup' ( ), for all treatments in Experiment 3 sprayed at the 4-6 trifoliate leaf stage on 12 July 2016, at Ashley Dene, Springston, Canterbury, New Zealand. Error bar is LSD for cultivar*herbicide interaction.

There was a herbicide*cultivar interaction (P=0.006) for the number of reproductive nodes per plant for subterranean clover at the November 2016 harvest. The number of potential reproductive nodes per plant was highest in flumetsulam treated 'Narrikup', MCPB treated 'Antas', and imazethapyr treated 'Narrikup', with 200+ reproductive nodes per plant (Figure 6.9).

MCPB, bentazone, and flumetsulam treated 'Antas' had 60-220 reproductive nodes, which were all the same as the unsprayed unweeded control with 136. Remaining herbicides had <43 reproductive nodes per plant, which was fewer (P=0.006) than the unsprayed unweeded control.

Treated 'Denmark' cultivars showed no difference in number of reproductive nodes compared with the unsprayed unweeded control with 13 reproductive nodes per plant.

Flumetsulam and imazethapyr treated 'Narrikup' had 200+ reproductive nodes per plant, more (P=0.006) than the unsprayed unweeded control which had 86 reproductive nodes per plant. All remaining treatments were not different to their unsprayed unweeded control.

The number of reproductive nodes per plant in treated 'Woogenellup' was not different to the control which had 68 reproductive nodes per plant (Figure 6.9).

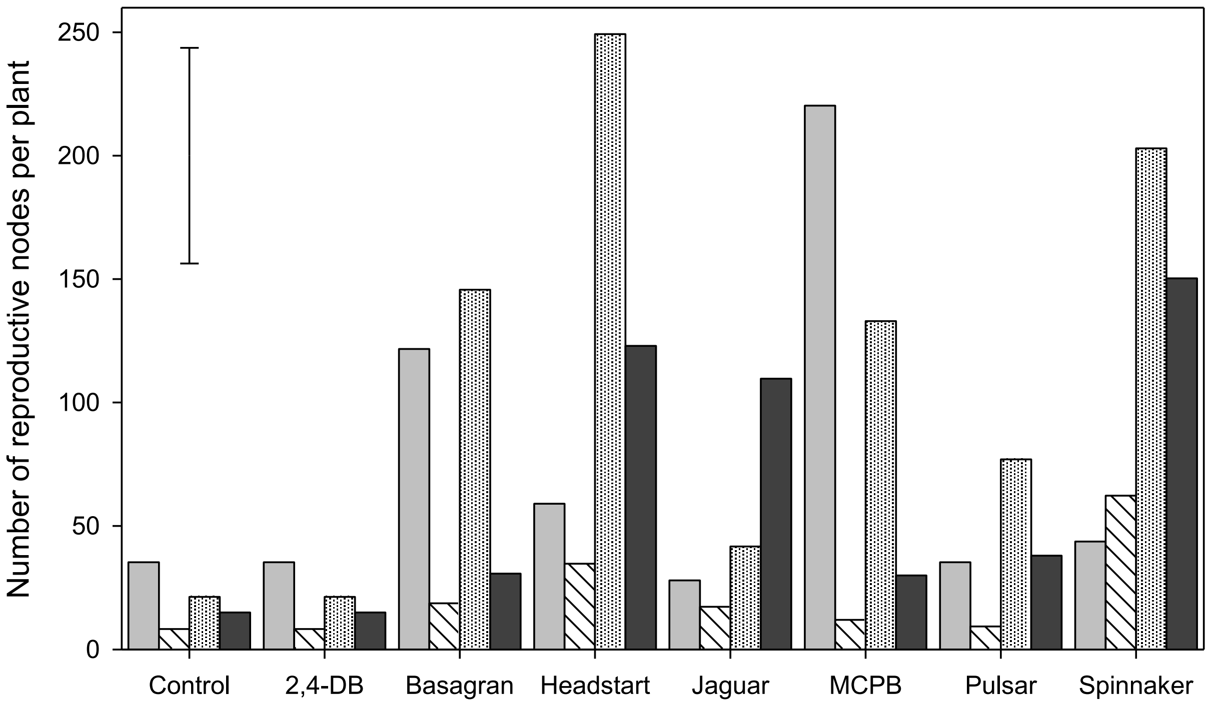

Figure 6.9 Number of reproductive nodes per plant on 13 November 2016 for 'Antas' ( ) 'Denmark' ( ), 'Narrikup' ( ), 'Woogenellup' ( ), for all treatments in Experiment 4 sprayed at the 4-6 trifoliate leaf stage on 12 July 2016, at C9BS Ashley Dene Farm, Springston, Canterbury, New Zealand. Bar is LSD for cultivar*herbicide interaction.

There was a herbicide*cultivar interaction (P=0.001) for the number of stage 3 burrs per plant for subterranean clover at the November 2016 harvest (Figure 6.10).

Stage 3 burr numbers of MCPB and bentazone treated 'Antas' were not different to the unsprayed unweeded control, which had 30 stage 3 burrs per plant. All remaining treatments had less than seven stage 3 burrs per plant, which was less (P=0.001) than the control (Figure 6.10).

Treated 'Denmark' cultivars were the same as the unsprayed unweeded control with six stage 3 burrs per plant (Figure 6.10).

Flumetsulam and imazethapyr treated 'Narrikup' with 60-75 stage 3 burrs per plant had more (P=0.001) than the unsprayed unweeded control, which had 31. 2,4-DB treated 'Narrikup' had two stage 3 burrs per plant, which was reduced (P=0.001) compared to the control. All remaining treatments were not different to the control.

Stage 3 burr numbers were not different for any treatments of 'Woogenellup' compared to the unsprayed unweeded control which had 16.

All 2,4-DB treatments had no stage 3 burrs per plant, except 'Narrikup', which had two stage 3 burrs per plant (Figure 6.10).

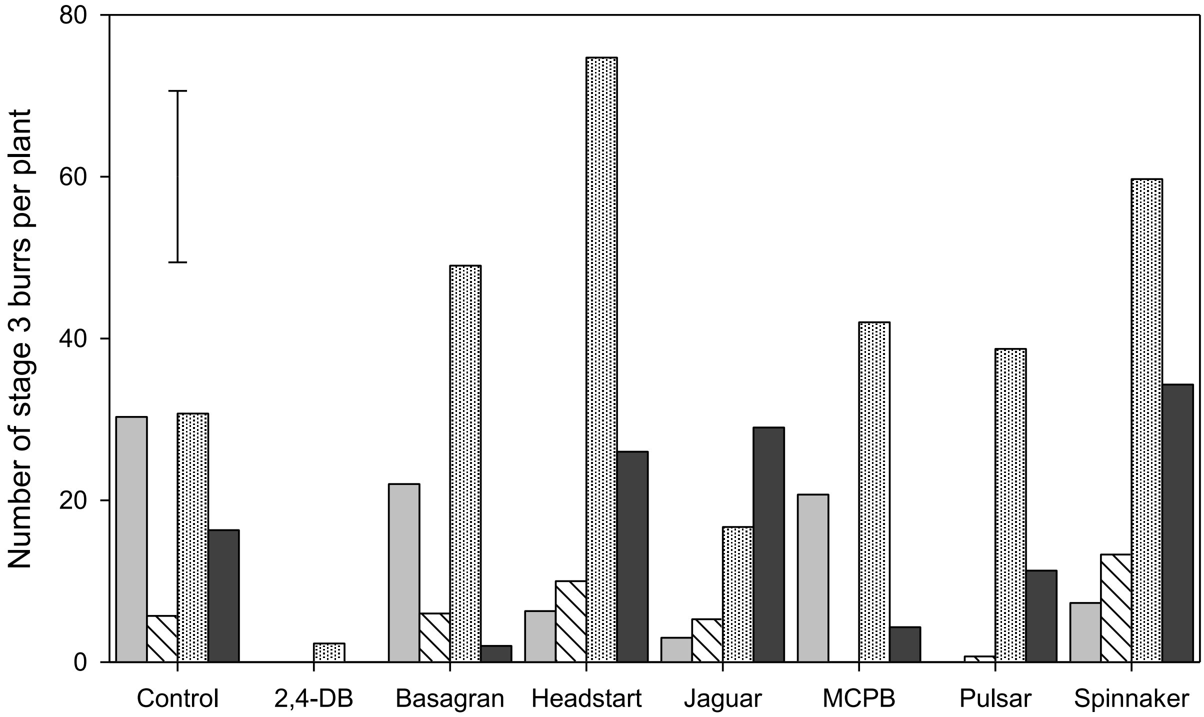

Figure 6.10 Number of stage 3 burrs per plant on 13 November 2016 for 'Antas' (■) 'Denmark' (▧), 'Narrikup' (▦), and 'Woogenellup' (■), for all treatments in Experiment 4 sprayed at the 4-6 trifoliate leaf stage on 12 July 2016, at C9BS Ashley Dene Farm, Springston, Canterbury, New Zealand. Error bar is LSD for cultivar*herbicide interaction.

6.3.7 Comparison between application timings

Paired t-tests of total yields for Experiments 3 and 4 showed that sown clover content was 328 ± 41 kg/ha higher (P<0.001) in Experiment 3 than Experiment 4 (Table 6.3).

Due to the earlier broadleaf weed control, Experiment 3 produced 379 ± 102 kg/ha less (P<0.001) total dry matter than Experiment 4, with 344 ± 80 kg/ha less (P<0.001) broadleaf weeds and 242 ± 103 kg/ha less (P<0.021) grass weeds (Table 6.3) for the 2016 growing season.

Table 6.3 Paired t-test results for 2016 dry matter yields (kg/ha) of Experiment 3 (1-2 leaf application time) – Experiment 4 (4-6 leaf application time) from 22 March – 11 November 2016 at Ashely Dene, Springston, Canterbury, New Zealand.

	1-2 leaf application	4-6 leaf application	Difference	T-Value	P-value
Sown clover	779 ± 64	451 ± 39	328 ± 41	7.98	<0.001
Resident Clover	956 ± 51	1082 ± 51	-126 ± 66	-1.92	0.057
Broadleaf weeds	652 ± 67	996 ± 92	-344 ± 80	-4.30	<0.001
Grass weeds	768 ± 59	1010 ± 81	-242 ± 103	-2.34	0.021
Total dry matter	3294 ± 92	3673 ± 94	-379 ± 102	-3.72	<0.001

6.3.8 Weed regeneration

Broadleaf weed regeneration six (Experiment 4) and seven (Experiment 3) months post application, measured on the 15 January 2017 was highest (P<0.001) in imazethapyr, flumetsulam, and MCPB treatments, with an estimated 10-40% weeds compared to the unsprayed controls. All other treatments had an average 60-70% weeds compared to the unsprayed controls, but were still reduced (P<0.001) in comparison. There was no difference in weed regeneration between experiments. Weed regeneration in 2,4-DB and MCPB appeared to be slower in Experiment 4, with 30-40% weeds compared to the control, while Experiment 3 had 50-60% weeds (Figure 6.11).

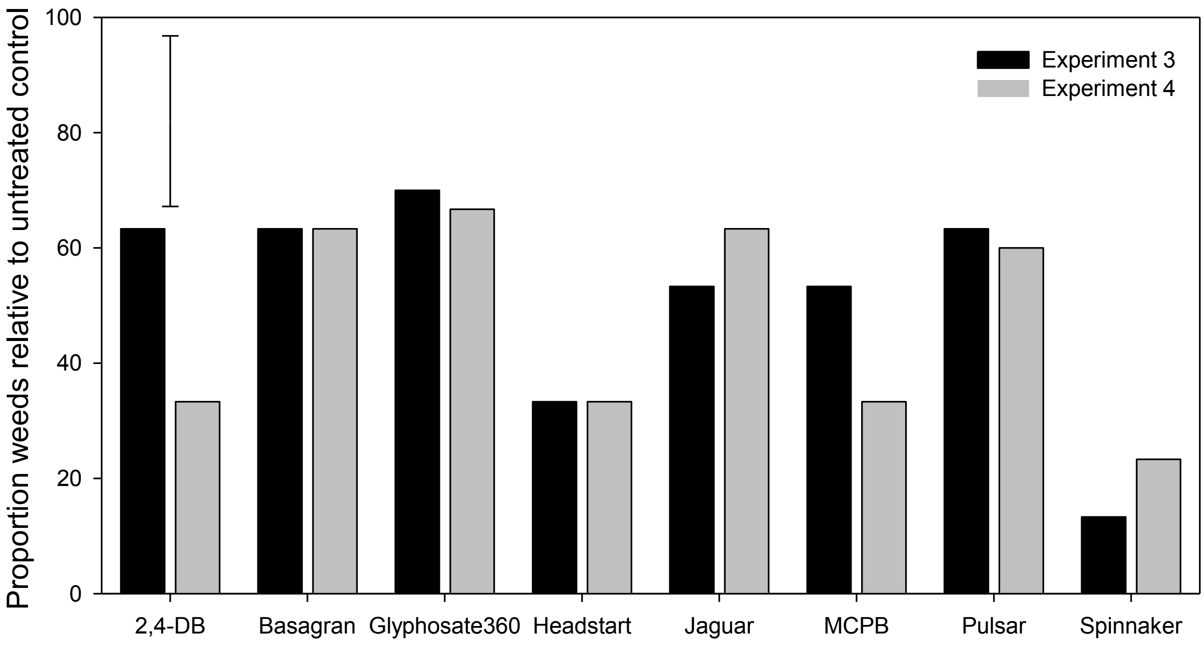

Figure 6.11 Proportion of weed regeneration relative to unsprayed unweeded control (100%) on the 15 January 2017, after treatment application on the 14 June 2016 for Experiment 3 (1-2 leaf application time), and 12 July 2016 for Experiment 4 (4-6 leaf application time) at Ashley Dene, Springston, Canterbury, New Zealand. Error bar is LSD for main effect of herbicide within experiments.

6.4 Discussion

6.4.1 Seedling establishment

Seedling numbers showed no difference between Experiments 3 and 4 immediately prior to herbicide applications on 12 July 2016 (Table 6.1). This suggests that any yield differences observed between the experiments resulted from a difference in response to the two application times. 'Monti' and white clover had fewer seedlings per m^2 than 'Antas', 'Narrikup', and 'Denmark', showing that they were less suited to persist in the dry autumn conditions.

6.4.2 Total yield potential

The total yield potential for this site can be seen in the total dry matter yield of the unsprayed unweeded control, which produced 5100 kg DM/ha from 22 March 2016 until 11 November 2016 (Figure 6.5). This was 50% broadleaf weeds (Figure 6.5), 500 kg/ha sown clover, 1200 kg/ha resident clover, 600 kg/ha grass weeds, and 100 kg/ha dead matter. This annual yield was reduced by herbicide treatment compared to the control. Total dry matter yields were reduced for all herbicides in the September harvest (Figure 6.3), but recovered after grazing and a month of regrowth, so all herbicides yielded the same as the unsprayed unweeded controls for the September – November growth period (Figure 6.4). This was also observed in Experiment 3 (Figure 5.8), but with 1000 kg DM/ha less broadleaf weeds, from the earlier application.

6.4.3 Difference between application times

Total dry matter yields were similar between experiments, however the later 4+ trifoliate leaf application had total season sown clover yields which were reduced by 40-50% lower in all treatments compared to those treated at the 1-2 trifoliate leaf stage. Control of broadleaf weeds and grasses was less effective in Experiment 4, with a mean 35% more broadleaf weeds, and 25% more grasses, while total dry matter yields were only 10% higher (Table 6.3). With no differences between experiments, the resident 'Woogenellup' was not so disadvantaged, but the cultivar has softer seed, and was pre-existing in the seed bank, so probably germinated earlier than the sown clovers, meaning it had a slightly longer growing season.

6.4.4 Glyphosate

Glyphosate was again completely phytotoxic to all clover, broadleaves, and grass weeds in this later 4-6 trifoliate leaf application time, with an EWRS score of 9.0 and ground completely bare prior to the first harvest in September. As in Experiment 3 (Section 5.4.3), all physical yields were assumed to be 0 kg DM/ha, and eliminated from yield analyses. With this result, glyphosate applied at this rate is not recommended for subterranean clover containing pastures. White clover was also susceptible, despite glyphosate being rated as safe on white clover. As discussed in Section 5.4.3, this is likely due to the poor season causing compounded stress (Rolston 1987), and white clovers inability to compete with the weeds to establish prior to treatment (Frame & Newbould 1986).

6.4.5 2,4-DB

2,4-DB was unsuitable for subterranean clover even when applied at this 4-6 leaf development stage. 2,4-DB treatments had the most visible phytotoxicity damage in surviving treatments, with an EWRS score of 5.8, higher than the unsprayed unweeded control, and all other herbicides (Figure 6.1). This again suggested that 2,4-DB treated plots would have the greatest yield reductions relative to their unsprayed unweeded control. Only 'Narrikup' showed a visible reduction in sown clover dry matter yields compared to the unsprayed unweeded control in the September harvest while all other cultivars were not different (Figure 6.2). Resident 'Woogenellup' yields were reduced, with less than half the yields seen in the controls (Figure 6.5). After grazing and recovery, the resident clover recovered to produce 850 kg/ha in all treatments while sown clover yielded a mean of 350 kg/ha, the same as the unsprayed unweeded control (Figure 6.5). With a season reduction in sown clover, and total dry matter yields (Figure 6.5), 2,4-DB treatment showed no advantage for the dry matter production of the pasture. In Experiment 3, sown clover yields for the season were only reduced for 'Antas' compared to unsprayed unweeded controls, which still yielded 150-950 kg DM/ha depending on the herbicide (Figure 5.7). This results shows that the later application had more prolonged effects on subterranean clover productivity, likely a consequence of reduced metabolic activity causing 2,4-DB accumulation (Section 2.5.1).

2,4-DB treated 'Narrikup' and 'Antas' had smaller plants in September (Table 6.2), and development was delayed by at least one runner stage for all cultivars compared to the unsprayed controls, despite application occurring at the recommended development stage (Figure 6.6). Plants had not recovered by the November harvest, with plants half the size of the unsprayed controls (Figure 6.7), and having fewer runners for all cultivars except 'Denmark' (Figure 6.8). These stunted plants also had fewer reproductive nodes and stage 3 burrs per plant than the unsprayed unweeded controls (Figure 6.9). However, the effect of 2,4-DB on total plant reproductive nodes was less pronounced than its effect on stage 3 burrs. 'Narrikup' with two stage 3 burrs was the only cultivar to have produced any by November 11. As discussed in Section 5.4.4, this substantial reproductive delay in response to 2,4-DB treatment is a cause for concern. While 2,4-DB reduced broadleaf weeds by 50% to 1300 kg/ha (Figure 6.5), this was the smallest reduction. When considered with the combined sown and resident clover yields, and the developmental impact; subterranean clover treated with 2,4-DB at the 4-6 trifoliate leaf stage was as disadvantaged by treatment application as it was by the weed ingress in the controls. Further to this, space created by removal of weeds remained unoccupied, showing 2,4-DB treatment of subterranean clover at the 4-6 trifoliate leaf stage in a late starting season was ineffective for weed control if the purpose was increasing the subterranean clover content of a pasture.

Plant development was delayed in all cultivars in 2,4-DB treatments across both treatment application times. Due to the low temperatures, and low growth activity of plants at the time of application, 2,4-DB treated plants were likely over exposed to auxin for some months. They were still showing signs of excessive vegetative growth in leaf axils often with supernumerary leaflets, in 4-10 leaflets, rather than the standard trifoliate at the final harvest in November 2016, 4 or 5 months post treatment application depending on the application timing (Plate 5.8). 2,4-DB treated plants were also obviously smaller and showed less vigour than their unsprayed unweeded control counterparts for all cultivars.

6.4.6 MCPB

MCPB treatments produced one of the smallest broadleaf weed reductions, with only a 55% reduction compared to the unsprayed unweeded control (Figure 6.5). An average EWRS score of 4.8 for MCPB treated plots also suggests phytotoxicity damage to the subterranean clover (Figure 6.1), and some yield decreases compared to unsprayed unweeded controls were expected. Average sown clover yields were lower compared to their unsprayed unweeded controls for the whole season, while resident clover yields were suppressed in the September harvest (Figure 6.3), but recovered to be not different by November (Figure 6.4). While treated resident 'Woogenellup' was able to recover, sown cultivars were too vulnerable during weed ingress, and failed to thrive after treatment. This left them unable to take advantage of the space provided by the broadleaf weed control.

There was also an effect of MCPB on plant productivity, with MCPB treated 'Narrikup' and 'Monti' reduced in size in September (Table 6.2), and all cultivars 100 mm smaller than unsprayed controls by early November (Figure 6.7). Despite this decrease in size, MCPB showed no effect on plant reproductive development in September or November (Figure 6.6, Figure 6.8, Figure 6.9, and Figure 6.10). Based on these results, MCPB treatment at the 4-6 trifoliate leaf stage has an impact on plant growth, but no adverse effects on the rate of development and seed set, and can provide some weed control. Despite this, the potential impact of extended weed ingress on the establishing subterranean clover plants, and the low level of weed control provided compared to other herbicides must be considered before treatment time is decided.

Compared to Experiment 3, where sown clover yields were unaffected by herbicide (Section 5.4.5, and Figure 5.7), this application time had an adverse effect on the yield potential of the sown subterranean clover. Experiment 3 also had a reduction in plant diameter, and a response in late development, where 'Antas' and 'Woogenellup' had reduced numbers of reproductive nodes and stage 3 burrs per plant. Based on these results, MCPB applied early is advantageous for yields, but can potentially compromise future persistence. However, when applied late, despite no effect on late reproductive development, yields are impacted, which will also likely affect future persistence, as lower plant densities also provide a decreased seed set (Smetham 2003).

6.4.7 Bentazone

Bentazone was again the only treatment with an EWRS score showing no visible difference to the unsprayed unweeded control. The consequence was that sown and resident clover yields for all bentazone treatments were not different to their unweeded unsprayed controls on both harvest dates (Figure 6.2 andFigure 6.4), and total dry matter yields for the season were the closest to the unsprayed unweeded control (Figure 5.8). With a 70% reduction in broadleaf weeds, weed control was not as effective as some of the other treatments, but as in Experiment 3 (Section 5.4.6) phytotoxicity to subterranean clover was not apparent, especially for 'Antas' which shows herbicide sensitivity (Figure 5.7). With a spray date four weeks later than Experiment 3, Experiment 4 treatments were applied in an air temperature of 6.6 ˚C on a warm fine day, and the average 2 cm depth soil temperature was 4.7 ˚C, well below the temperatures recommended for optimal bentazone performance (Section 2.5.2.1). Development at all monitored stages was not adversely affected by bentazone for all cultivars (Table 6.2, and Figure 6.6, Figure 6.7, Figure 6.8,Figure 6.9, and Figure 6.10).

Bentazone applied at the 1-2 trifoliate leaf stage in Experiment 3 reduced the number of reproductive nodes and stage 3 burrs for 'Antas', and delayed the rate of reproduction for 'Woogenellup' (Section 5.4.6). The early application also provided a 650 kg DM/ha increase in sown clover yields compared to the unsprayed unweeded controls, and combined clover yields of 2000 kg DM/ha, compared to the 1500 in Experiment 4. Remaining broadleaf weeds were 600 kg DM/ha, suggesting no difference in the levels of weed control despite the later application time.

Bentazone has potential as a herbicide for subterranean clover containing pastures, with no reduction in clover yields, and no effects of plant development rates when applied when subterranean clover was at the 4-6 trifoliate leaf stage. However weed control efficacy was likely reduced by the mid-winter application time, at temperatures below 10 °C and earlier germinating seasons may see increased weed control due to higher photosynthetic activity (Section 5.4.6). Applications in warmer temperatures could also see a potential increase in phytotoxic effect on the subterranean clover component.

6.4.8 Bentazone + MCPB

Bentazone + MCPB is a mixed formulation of MCPB (Section 6.4.6) + bentazone (Section 6.4.7), and clover responses were expected to be similar to those found in their treatments.

Bentazone + MCPB treated clover had an average EWRS score of 2.9, which meant in the September harvest, all cultivars yielded the same as their unsprayed unweeded controls except 'Narrikup' (Figure 6.2). By November, there was no difference in sown clover yields (Figure 6.4). Sown and resident clover yields for the season were not different to the unsprayed unweeded control, but total dry matter yields were reduced as a result of the 1600 kg/ha broadleaf weed reduction, and the subterranean clover failing to occupy the space provided. In September, bentazone + MCPB treated 'Denmark' and 'Monti' were larger than their controls suggesting some advantage to the weed control, and there were no differences in development compared to the unsprayed unweeded controls (Figure 6.6). However in November, 'Antas' had a reduction in the number of runners (Figure 6.8), reproductive nodes (Figure 6.9), and stage 3 burr numbers per plant (Figure 6.10).

Experiment 3 and 4 both had combined clover yields of 1500 kg DM/ha suggesting there is no effect of bentazone + MCPB application time on clover yields, however, those sprayed earlier were unable to take advantage of the space provided to them, rendering the early application pointless in this situation.

The *brachycalycinum* 'Antas' was susceptible to the bentazone + MCPB active ingredient mixture of Bentazone + MCPB regardless of development stage at application timing, as this susceptibility was also observed in Experiment 3, (Section 5.4.7). No other cultivars showed signs of affected development, showing that 'Woogenellup', with no reduction in stage 3 burrs (Figure 6.10) was less sensitive to developmental changes caused by bentazone + MCPB when treated at a later growth stage. Based on the results for applications of bentazone (Sections 5.4.6 and 5.4.7) and MCPB (Sections 5.4.5 and 6.4.6), MCPB is more likely causing the development delays. With the cooler temperatures at application (Figure 5.1), this could be a response to accumulation of synthetic, unstable auxin within the plant (Section 2.5.1) and in warmer conditions, effects observed in these experiments may not occur.

The lack of favourable response to bentazone + MCPB suggests it should not be used as a herbicide option in subterranean clover containing pastures unless other options are unavailable.

6.4.9 Bromoxynil + diflufenican

Bromoxynil + diflufenican treated sown clover, with an average EWRS score of 2.9 (Figure 6.1) indicated an expected reduction in yields, or no difference to the unsprayed unweeded controls. Early season yields of 'Antas' and 'Narrikup' were reduced while all other cultivars showed no phytotoxicity effect in sown clover yields (Figure 6.2). Bromoxynil + diflufenican also reduced September yields of resident clover to less than 140 kg/ha (Figure 6.3). After grazing and recovery, mean yields of sown clover were reduced compared to the 550 kg/ha in the unsprayed unweeded control, but resident clover yields were unaffected, totalling a combined 1000 kg/ha of subterranean clover for the season (Figure 6.4). Broadleaf weeds were reduced by 70%, while grass weeds were visually higher, but due to high yield variation between plots, relatively unaffected compared to the control (Figure 6.5). As a result of high broadleaf weed control, but phytotoxicity causing reductions in the sown clover component of the pasture, total season dry matter yields were 25% lower than the unsprayed unweeded control (Figure 5.8).

Bromoxynil + diflufenican treated plots showed no visible effect on subterranean clover growth and early development compared to the unsprayed unweeded controls in September (Table 6.2, and Figure 6.6). However by November, plant diameters were reduced by 100 mm (Figure 6.7), and all sown cultivars had fewer runners per plant, with only the resident 'Woogenellup' unaffected (Figure 6.8). 'Antas' also had fewer reproductive nodes (Figure 6.9), and stage 3 burrs per plant (Figure 6.10) while all other cultivars were unaffected despite reductions in runner numbers.

Bromoxynil + diflufenican applied at the 1-2 trifoliate leaf stage had no adverse effects on the sown subterranean clover yields, (Figure 5.7), and mean combined clover yields were 60% higher at 1750 kg DM/ha than the later 4-6 trifoliate leaf stage application. Broadleaf weed control was also higher in Experiment 3, with less than 350 kg DM/ha remaining compared to the 750 kg DM/ha in Experiment 4. Despite these increased yields, plants with early bromoxynil + diflufenican applications had reduced stage 3 burrs (Figure 5.13), suggesting an impact on the rate of growth, and a potential impact on the future persistence of the pasture.

With the early effect on subterranean clover growth when applied late, and long term reductions in plant size, bromoxynil + diflufenican is not the first choice of herbicide. However, because it does not affect grass growth, it could be an option in pastures that contain grasses not tolerant of other herbicides. The developmental delay observed in 'Antas' at the late stage would have to be considered however, as there is the potential for significant damage to the long term success of the pasture if the subterranean clover plants are unable to reproduce.

6.4.10 Flumetsulam

Flumetsulam treatments had an average EWRS score of 3.2, higher than the unsprayed unweeded control (Figure 6.1). This low EWRS score was associated with September harvest sown clover yields which were not different to the unsprayed unweeded controls, except for 'Narrikup' being lower (Figure 6.2). After grazing, average sown clover yields of all cultivars recovered and were not different to the unsprayed unweeded controls (Figure 6.4). Sown and resident clover yields for the season were not different to the unweeded unsprayed control (Figure 6.5). Broadleaf weeds were controlled by 95%, which was the same as in Experiment 3 (Section **Error! Reference source not found.**), while grass w eeds were unaffected. There was a 2000 kg/ha reduction in total dry matter yield (Figure 6.5) due to the subterranean clover leaving the space provided unoccupied.

In September, treated ‘Antas’ and Denmark’ plants were larger than their unsprayed unweeded controls (Table 6.2), and there was no effect on early plant growth (Figure 6.6). By November, while plant diameters of all cultivars were not different to the controls (Figure 6.7), ‘Denmark’ had about five more runners per plant (Figure 6.8). Despite no change in runner number, flumetsulam appears to have advanced the rate of development of ‘Narrikup’, which had more reproductive nodes (Figure 6.9) and stage 3 burrs per plant than the unsprayed unweeded control (Figure 6.10). Meanwhile, ‘Antas’ had fewer stage 3 burrs than the unsprayed unweeded control, but no fewer reproductive nodes per plant (Figure 6.9). Based on these results, sown ‘Narrikup’ and ‘Denmark’, and the resident ‘Woogenellup’ subterranean clover cultivars are tolerant of flumetsulam treatment, while ‘Antas’ again appeared susceptible with a phytotoxicity response of mild development delay, despite not having a difference in dry matter yields.

In both application times, ‘Antas’ showed a reduction in stage 3 burr numbers, and ‘Narrikup’ had increases (Figure 5.13, and Figure 6.10). This result suggests that flumetsulam was safe on subterranean clover from an early development stage, and this tolerance was a function of cultivar, rather than development stage. There was no difference in the level of weed control achieved, but combined clover yields were 2000 kg DM/ha in Experiment 3 (Figure 5.8), 25% higher than the later application (Figure 6.5), and ‘Antas’ and ‘Narrikup’ yielded 1000 kg DM/ha higher when sprayed at the 1-2 leaf stage (Figure 5.7).

With high levels of weed control regardless of application time and temperature, along with no long term phytotoxicity effects on ‘Denmark’, ‘Narrikup’, and the resident ‘Woogenellup’, flumetsulam is a viable option for use on subterranean clover in New Zealand, and is recommended on label for (Zelam 2016).

6.4.11 Imazethapyr

Imazethapyr had an average EWRS score of 3.2, which indicated potential decreases in yields of sown clover, and this corresponded with a yield decrease for ‘Antas’ and ‘Narrikup’ in the September harvest, while all other cultivars were not different to their unsprayed unweeded controls (Figure 6.2). Resident clover yields were also reduced in the September harvest compared to the controls, suggesting a phytotoxic response to imazethapyr treatment. Post-grazing the sown clovers recovered, with average sown clover and resident clover yields not different to the unsprayed unweeded controls (Figure 6.4). Total season yields for clover treated with imazethapyr were not different to the unsprayed unweeded control, while broadleaf weeds were reduced by 95% (Figure 6.5). However, total dry matter yields were reduced, and the subterranean clover did not manage to occupy the provided space and increase yields. There was no early effect of imazethapyr on subterranean clover plant size or development in September (Table 6.2 and Figure 6.6). By November, 'Denmark' had an increase in the number of runners per plant (Figure 6.8), however this didn't cause an increase in reproductive nodes (Figure 6.9). Meanwhile, 'Antas' had a reduction in the number of runners, reproductive nodes and stage 3 burrs per plant (Figure 6.10). These results show that 'Antas' was more susceptible to developmental delays in reproductive stages, and should not be treated with imazethapyr if the pasture is intended for subsequent regeneration. As was discussed in Sections 5.4.13 and 6.4.10, while remaining cultivars were not affected by imazethapyr, it was too cold for them to grow and compensate for the reductions in broadleaf weed yields. As a result, mid-winter applications are not ideal, and show no increase in establishment compared to unsprayed unweeded controls, so should only be done if weeds are unpalatable, or are likely to be detrimental to the future persistence of the pasture.

With a mean 2300 kg DM/ha combined clover yields, clover treated early with Spinnaker was 40% more productive (Figure 5.8) than the later application (Figure 6.5), and sown clover yields were increased compared to controls for 'Antas' and 'Narrikup' (Figure 5.7). Only 'Antas' had a reduction in the number of stage 3 burrs per plant (Figure 6.10), again suggesting that *brachycalycinums* have greater susceptibility to developmental delays in response to herbicides.

With no adverse effects on the other cultivars, and higher yields in Experiment 3, imazethapyr can be applied at the 1-2 trifoliate leaf stage without reductions in yields or effects on pasture persistence, and weed control is achieved early in the season.

6.4.12 White clover

'Kopu II' white clover consistently yielded a mean 600 kg/ha less than sown 'Antas' and 'Narrikup' in this experiment (Figure 6.2, and Section 6.3.5), with season yields of less than 200 kg/ha, which were not different to 'Denmark' and 'Monti'. This was also not different to Experiment 3, with 300 kg DM/ha (Figure 5.7).

6.4.13 Subterranean clover

Sown subterranean clover yields for treatments applied at the 4-6 trifoliate leaf stage showed a mean 40% reduction in yields compared to Experiment 3 (Table 6.3). Results show a reduced sown cultivar*herbicide interaction in response to herbicide application compared to what was observed in Experiment 3 (Figure 5.7). Sown clover yields for herbicides applied at the 4-6 trifoliate leaf stage had little variation in total yields among cultivars and herbicides (Figure 6.2), and these differences were only present in the early harvest. By the later harvest, there were no differences between sown cultivars for herbicide treatments (Figure 6.4), but yields were also lower than those in Experiment 3.

When treated at the 4-6 trifoliate leaf development stage, the sown subterranean clover plants did not take advantage of the gaps provided and fill them out, causing broadleaf weed reductions, but no sown clover, resident clover, or grass increases compared to the unsprayed unweeded controls. If plants had germinated earlier, subterranean clover treated late at the 4-6 trifoliate leaf stage may have recovered from treatment earlier, and produced higher yields than subterranean clover treated at the 1-2 leaf stage, with less long term phytotoxicity effects. This should be investigated in a subsequent season to further isolate environmental effects from cultivar responses.

Based on the combined dry matter and development results of this experiment, spraying at the later 4-6 leaf development stage in a season with a delayed break had no advantage for the subterranean clover component, or total dry matter yields of the site in the early part of the season. Unsprayed unweeded 'Narrikup' was the highest yielding in September at 700 kg/ha, and only bentazone treated 'Narrikup' was the same as this. All other cultivars and herbicides were lower yielding (Figure 6.2). After grazing and recovery, there was no cultivar*herbicide interaction or main effect for sown clover yields (Figure 6.4).

This result suggests that weed control in mid-winter may not be worth the cost, having the potential to damage the reproductive success of the subterranean clover through reduced plant densities. Further to this if weeds are palatable, they provide feed and can be controlled with grazing management, whereas if they are killed, their removal substantially reduces the total available feed (Figure 6.5).

6.4.14 Environment

A lack of rainfall delayed the experiment, with plants not reaching the 4-6 trifoliate leaf stage until July. With herbicide applied in mid-July, average air temperatures were lower than the recommended lower limit of 10 °C at 6.6 ˚C (Figure 5.1), which limited the ability of the sown subterranean clovers to grow and occupy the spaces provided by the weed control in this experiment. With the delay in herbicide application until subterranean clover plants were at the 4-6 trifoliate leaf stage, broadleaf weeds were given more time to grow, and so were more developed at the time of application, and potentially more resistant to treatment. As weeds are left, they become harder to eliminate as effectively as in their early seedling stage. So while clover may not have been affected by the herbicide treatment, it was subject to heavy competition from broadleaf weed and grass seedlings, reducing its success in persistence. With average season sown clover yields of <600 kg DM/ha for all treatments (Section 6.3.5), the weed competition during the early growth period would likely have a strong impact on final yields, and the late weed control, while effective with 50-95% control for all herbicides (Figure 6.5), was too late to recover the yield loss.

6.4.15 Weed regeneration

There was no visual difference in weed regeneration between experiments. Weed populations in mid-January 2017 for imazethapyr, flumetsulam, and MCPB treatments, had an estimated 10-40% weeds compared to the unsprayed controls (Figure 6.11). All other treatments had an average 60-70% weeds compared to the unsprayed controls, but were still reduced ($P<0.001$) in comparison. Weed regeneration of 2,4-DB and MCPB appeared to be slower in Experiment 4, with 30-40% weeds compared to the control, while Experiment 3 had 50-60% weeds.

6.5 Conclusions

Subterranean clover treated with herbicides at the 1-2 trifoliate leaf stage had higher yields than subterranean clover treated at the 4-6 trifoliate leaf stage. Late season, mid-winter weed control was not as effective as earlier applications, as the subterranean clover was affected by weed ingress from early in establishment, and could not recover after weeds were eliminated. Alongside this, the weeds were not as well controlled primarily due to the later broadleaf weed growth stage, and also potentially the cooler weather conditions at application reducing the efficacy of the herbicides.

Imazethapyr and fumetsulam were the most promising herbicides for use on subterranean clover with no adverse effect on plant development. Imazethapyr was also safe on establishing subterranean clover-cocksfoot pasture mixes, allowing increases in the sown clover component, with no yield reductions where the subterranean clover cultivar was tolerant. Bentazone is an option where application can occur before temperatures drop. 2,4-DB should not be recommended as an option for subterranean clover containing pastures due to its lack of weed control compared to other herbicides, and adverse effects on potential seed set of all cultivars.

7 GENERAL DISCUSSION

The aim of this thesis was to identify suitable commercial options for broadleaf weed control in subterranean clover-containing pastures in New Zealand. This involved evaluation differences in responses of subterranean clover cultivars to the different mechanisms of action, and identification of potential causes of differences for increased understanding and further experimentation (Figure 7.1). This chapter discusses the results from all experiments, their implications for management use in New Zealand dryland pastoral farming systems, and identifies avenues for further areas of investigation.

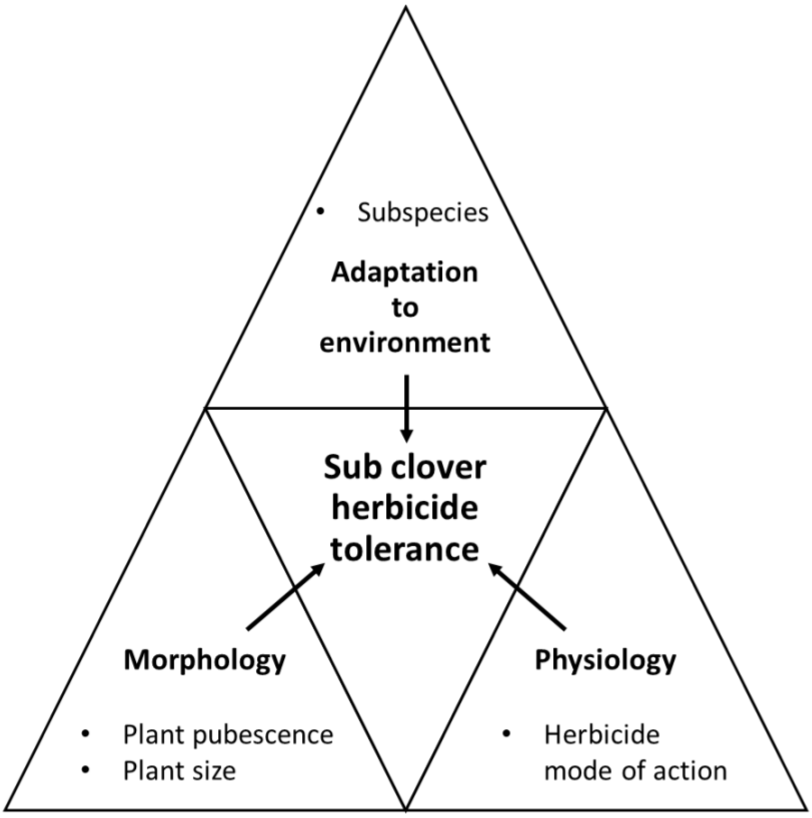

Figure 7.1 Diagram of synthesized findings which influence subterranean clover cultivar herbicide tolerance.

7.1 White clover

White clover performance was poor throughout all experiments, with mean yields 1000 kg/ha lower than the highest producing subterranean clovers. This result, in a late germinating year when both the white and the subterranean clover were under moisture stress (Experiments 1 and 2, Sections 3.4.3, and 4.4.3), or had a reduced growing season (Experiments 3 and 4, Sections 5.4.13, and 6.4.14) highlights the requirement for an alternative legume option to fill the early spring lactation feed gap in these areas.

7.2 Environment

Results for all experiments confirmed the existence of a cultivar*herbicide interaction for subterranean clover herbicide tolerance. This was especially highlighted in Experiment 1 where 13 subterranean clover cultivars were assessed for imazethapyr tolerance, but only three; 'Narrikup', Whatawhata', and 'Woogenellup' showed visual tolerance and high yields (Figure 3.4, Figure 3.8, and Figure 3.9) due to higher plant pubescence (Figure 3.5). For this experiment, 'Denmark' and 'Antas' were the only other subterranean clover cultivars to yield higher than 1000 kg sown clover/ha, but had greater visual herbicide damage than Narrikup', 'Whatawhata' and 'Woogenellup' (Figure 3.9). The yield of 'Antas' was lower than seen in other environments (Lucas *et al.* 2015; Mills *et al.* 2014). With the application of imazethapyr to Experiments 1 and 2 on the 7th of April followed by a month of no rainfall, plants which were already stressed by moisture deficits, had this stress exacerbated by herbicide application (Plate 3.3 and Plate 3.4). Despite the three month delay in germination, 'Antas' treated with imazethapyr in Experiment 3 yielded higher at 1750 kg DM/ha than in Experiments 1 and 2 with 1250-1500 kg DM/ha. The morphological response of 'Antas' to imazethapyr treatment in Experiment 3 was also different. These plants were noticeably larger than control plants, and showed no signs of phytotoxicity (Plate 5.5, and Plate 5.6). With plant susceptibility to herbicide damage increased by moisture stress (Cobb & Reade 2010; Roberts 2000) there was a significant environmental impact across all early sown experiments (Figure 7.1).

7.3 Mechanisms of tolerance

Experiment 1 found that plant pubescence afforded greater visual tolerance to imazethapyr treatment (Figure 3.5), which was in turn correlated with greater dry matter yields (Figure 3.9). This advantage afforded by plant pubescence was of particular benefit to 'Narrikup' and 'Woogenellup', and was seen in the consistently higher yields across all experiments (Figure 3.8, Figure 4.5, Figure 5.7, and Figure 6.2). With all herbicides applied with a wetter-spreader adjuvant, relative rates of permeability and droplet stickiness are expected to be consistent among experiments, within cultivars (Sections 2.3 and 3.2.7.1). As herbicide absorption is not increased by stress, but metabolic rates are (Roberts 2000), the decreased tolerance which was observed by susceptible cultivars in Experiment 1 was not a response to the increased penetration of the active ingredient. The increased phytotoxicity of the glabrous plants compared to what was expected, can potentially be attributed to a reduced metabolic rate, allowing increased active ingredient accumulation within the stressed plants as a result of increased moisture stress (Roberts 2000). Pubescent plants avoided cumulative stress by avoiding herbicide uptake. With the potential correlation of tolerance with plant pubescence (Figure 3.5), a focus for subterranean clover breeding programs and selection for resilient pastures could in the future concentrate on increased levels of plant pubescence, especially in leaves and petioles. With this focus, farmers may have more certainty when selecting and using chemical weed control methods in areas and years with lower rainfall.

7.4 Cultivar tolerance

Subspecies *subterraneum* cultivars showed the most tolerance across the experimental sites in this study. 'Narrikup' was the most tolerant cultivar across all experiments, with higher yields for all imazethapyr treatments, and little change in yields from the unsprayed unweeded controls for all other herbicides in Experiments 3 and 4 (Figure 5.7 and Figure 6.5). 'Woogenellup', sown in Experiment 1, and as resident clover in Experiments 3 and 4 also showed herbicide tolerance with high yields (Figure 3.8, Figure 5.8 and Figure 6.5). However, there may have been some effect of herbicides on successful seed set with the early application treatment (Figure 5.13), which was not observed with the later application (Figure 6.10).

The subspecies *brachycalycinum* cultivar 'Antas', which has been found to yield well in New Zealand environments (Lucas *et al.* 2015; Mills *et al.* 2014) had consistently low yields through all experiments, however dry matter yields in the unsprayed unweeded controls of Experiments 3 and 4 were greater than those in Experiments 1 and 2, despite the three month reduction in the growing season. This variation in yield suggests that 'Antas', was susceptible to drought stress at germination, especially when the water deficit was prolonged, as it was in this season (Figure 3.1). With later emergence in Experiments 3 and 4 at Ashley Dene, we would have expected diminished yields, but adequate winter rainfall at (or post) emergence (Figure 5.1) meant 'Antas', with its high yield potential, was able to compensate for the shortened growing season. This also translated into higher than expected yields for treated 'Antas' in Experiments 3 and 4, which illustrate the effect of season and environment on plant herbicide tolerance (Figure 7.1).

All *yanninicum* cultivars ('Monti', 'Napier' coated, and 'Trikkala') sown yielded less than 500 kg DM/ha across all experimental sites (Figure 3.6, Figure 5.7, and Figure 6.2), which suggests that they were not suited for the environmental conditions in these cold and/or dry areas of New Zealand.

The variation in results for these subspecies highlights a potential mechanistic difference in how the subspecies of subterranean clovers respond to herbicides (Figure 7.1). This difference could be due to their differences in edaphic adaptation (Section 2.1), but was not confirmed in these experiments and would require more in depth study.

7.5 Plant development

Due to the annual life cycle of subterranean clover (Section 2.1.1), reproductive development is essential to ensure the preparation of the pasture was not a wasted expense. If plants are producing mature seed at a slower rate than normal, the productivity and persistence of the pasture for future years, which is reliant on an ample seed set, is not ensured. Herbicides that delay this, such as 2,4-DB (Sections 5.4.4, and 6.4.5) are therefore unsuitable for use on subterranean clover. 2,4-DB delayed plant development across both application times, which showed that tolerance does not increase with growth, as in white clover (Rolston 1987). Due to the low temperatures, and low growth activity of plants at the time treatment was applied, 2,4-DB treated plants were likely over exposed to auxin for some months. They were still showing signs of excessive vegetative growth in leaf axils often with 4-10 supernumerary leaflets, rather than the standard trifoliate at the final harvest in November 2016, 4 or 5 months post treatment application depending on the application timing (Plate 5.8). 2,4-DB treated cultivars were also smaller and showed less vigour than their unsprayed unweeded control counterparts. No other herbicide had such broad effects on plant development.

The higher clover yields in imazethapyr and flumetsulam (Sections 5.4.9, 5.4.10, 6.4.10, 6.4.11 and 7.6) herbicide applications, with their reduction of broadleaf weeds by >95% compared to the unsprayed unweeded controls, lack of apparent effect on growth and reproductive development, and relative suppression of grass weeds compared to older recommended clover-safe herbicides such as 2,4-DB and MCPB recommends them as potential options to aid in the establishment year of a subterranean clover containing pasture. However, with their extended soil residual, use of bentazone may be preferable if temperatures are between 10 and 25 °C, and warm enough to allow optimal action, although this has the potential to result in increased subterranean clover phytotoxicity (Sections 5.4.6, 6.4.7, and 7.6).

7.6 Herbicides

The ALS inhibiting herbicides flumetsulam and imazethapyr (Sections 5.4.9, 5.4.10, 6.4.10, and 6.4.11) showed the greatest subterranean clover yield increases and weed control under all experimental conditions and application times. They achieved high levels of weed control irrespective of weed size and age at the time of application, and subterranean clover which yielded higher than 100 kg/ha in the early September harvest recovered after heavy grazing to yield the same as, or higher than the subterranean clover component of the unsprayed unweeded controls. This shows that imazethapyr treatment was advantageous, eliminated the broadleaf weeds and increased the subterranean clover success, without affecting the long term productivity of the pasture. This, combined with the findings of Experiments 3 and 4 which showed that if necessary, subterranean clover can be treated at the early seedling 1-2 trifoliate leaf development stage with no effect on yield (Figure 5.7 and Figure 6.5) shows that imazethapyr was an option for weed control to aid in establishment of subterranean clover pastures, so long as the cultivar has tolerance.

Bentazone (Section2.5.2.1) was the other promising herbicide. However its activity is considered optimal at temperatures between 10 and 25 °C, which was not the case in these experiments (Figure 5.1, and Sections 5.4.6 and 6.4.7), and unlikely to be the case in autumn when establishing subterranean clover pastures in New Zealand. With warmer temperatures, weed control would likely have been greater, however subterranean clover damage may also increase with faster action. With warmer temperatures, subterranean clover growth and metabolism would also be increased, likely increasing the levels of phytotoxicity damage observed. However it may allow plants to detoxify at a faster rate, potentially causing reduced damage although further observations in such conditions are required.

7.7 Pasture mixtures

Experiment 2 showed that subterranean clover cultivars which were tolerant of imazethapyr herbicide treatment can be sown in conjunction with cocksfoot. Suppression of the cocksfoot in the early seedling stage aids the establishment of the subterranean clover while eliminating weeds from the sward (Figure 4.5). This interaction allowed subterranean clover/cocksfoot pastures to establish successfully, with the subterranean clover component comprising more than 50% of the pasture, rather than the 25% observed in the unsprayed controls (Sections 4.3.3.3, 4.4, 4.5). This is particularly advantageous, as studies of subterranean clover-cocksfoot mixes by Lucas et al. (2015) in the same environment had ~30% broadleaf weeds and achieved similar total dry matter yields in the 2015 season to those in Experiment 2 in the 2016 season (Figure 4.5).

Where cocksfoot was sown with tolerant 'Narrikup' and treated with imazethapyr, 6000 kg DM/ha of high quality early spring feed available for lactation was produced, and 50% was the sown subterranean clover (Figure 4.5). This results shows that compared to the imazethapyr treated white clover/cocksfoot mixture which produced 3000 kg DM/ha, with only 30% sown clover, a tolerant subterranean clover cultivar can produce three times the amount of high quality legume for early spring, even in a year with moisture stress.

7.8 Application times

Investigation into herbicides for subterranean clover, and appropriate application times has shown that establishment success is aided by an early treatment application through early elimination of weeds (Scammell & Ronnfeldt 1998). This reduced sward competition for resources from grasses and weeds, which increased clover seed germination and establishment in the gaps created by the herbicide treatment while environmental conditions were suitable. A later application showed lower reductions in broadleaf weeds and lower clover yields as a result of the prolonged early competition for resources. With the application of a successful herbicide, weeds were eliminated from the sward. This changed the population dynamic to only the target, tolerant species from an early stage. This in turn reduced the competition between species for light, and changed early light ratio dynamics, with increased red light hitting clover seedlings, which increased their initial chances of success (Cressman *et al.* 2011). If weeds can be eliminated from the sward early in the growth cycle when at their ideal target stage for many herbicides, as in Experiment 3 with the 1-2 trifoliate leaf stage application time, competition for resources is ideally limited to only those desired species. This allows further germination if conditions are favourable, leading to increased plant densities and also results in greater growth to fill the gaps left by the dead weeds. The increase in plant density can act in the same way as weeds within the sward, decreasing the red light reaching all plants, leading to dense runner production and changes in plant diameters, explaining the reduction in pant diameters for all herbicide treated plants compared to the controls (Figure 5.10 and Figure 6.7). There is some confounding as to whether this change in diameters is a result of decreased light, alterations to biochemical pathways, or increased grazing pressure. Further investigated into these mechanisms is merited to increase understanding.

7.9 Future research

Another year of phytotoxicity, yield and development data in the same sites should be collected to observe effect of season. To confirm the advantage of pubescent cultivars like 'Narrikup' over glabrous cultivars like 'Antas', investigation into the effect of application rates on plant tolerance could provide useful insight. Further investigation into differences in responses of the subterranean clover subspecies is also advised, to determine if *subterraneums*, *brachycalycinums*, and *yanninicums* respond differently to treatment without the influence of environment. This could also be achieved using biochemical and molecular investigation to determine the mechanistic differences in subterranean clover species and cultivars for metabolic rates of the different active ingredients. Identification of this could provide rapid screening methods for herbicide tolerance in plant breeding.

Investigation into the effects of herbicide application on seed set and subsequent regeneration in New Zealand is important, especially in the case of flumetsulam, imazethapyr, and bentazone which have potential in subterranean clover containing pastures.

7.10 Conclusions

Herbicide tolerance in subterranean clover cultivars is a complex of environmental adaptation, physiological traits that confer barriers to penetration, and metabolic response to active ingredient uptake (Figure 7.1). There is a cultivar*herbicide interaction, and this was exacerbated by changes in available moisture, affecting plant metabolism rates. 'Narrikup' was the most tolerant subterranean clover cultivar evaluated, followed by 'Woogenellup'. This was hypothesized as a result of to decreased herbicide contact through greater leaf and petiole pubescence. As a result, plant pubescence should be investigated as a potential trait to prevent herbicide uptake by the non-target species. This could increase herbicide tolerance in marginal years when stress causes increased herbicide metabolism.

Across both application times, imazethapyr, flumetsulam, and bentazone were the most promising herbicide treatments, with minimal phytotoxicity damage and no yield reductions for sown or resident clover compared to the unsprayed unweeded controls. With warmer conditions, bentazone will likely have increased weed control, but this may also increase phytotoxicity damage. The effects of imazethapyr and flumetsulam should not change with temperature, and are safe on clover at both early and late seedling stages. As a result, they can be recommended for use in establishing subterranean clover pastures where tolerant cultivars are with no limitations of application time and temperature. In better years, where rainfall occurred earlier in autumn, the advantage might not be as pronounced, but if there are no adverse effects on yields for the season, early weed control is always an advantage.

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Zelam 2016 Flumetsulam Specimen Label Zelam Limited, Hudson Rd, New Plymouth, New Zealand

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Appendix

Table A.1 Agronomic data for Australian subterranean clover cultivars which have been sown in New Zealand (Lucas *et al.* 2015).

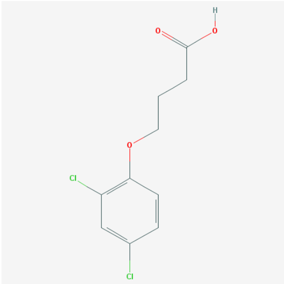
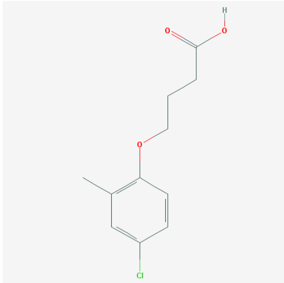
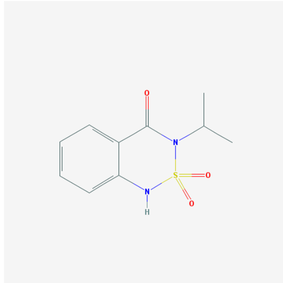
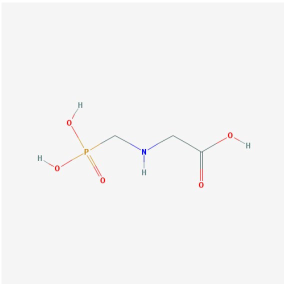
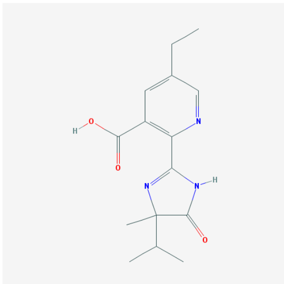
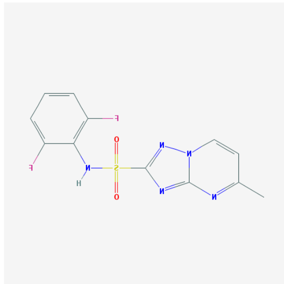
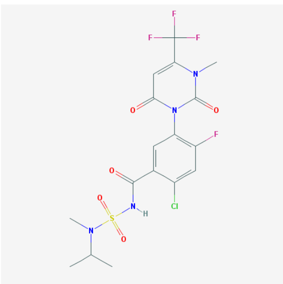
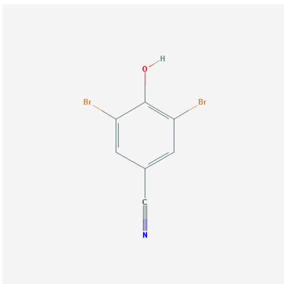
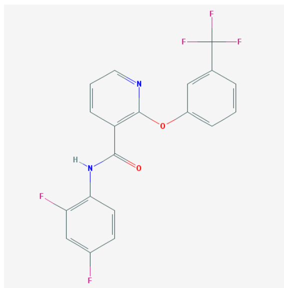
Cultivar	Year	Subspecies	Days to first flower	Minimum growing season length (months)	Burr burial rating (1-9)	Hard-seededness (1-10)	Seeds/m^2 sown at 10 kg/ha
'Mt Barker'	1900	S	137	7.5	3	1	120
'Tallarook'	1936	S	163	9	5	1	135
'Woogenellup'	1959	S	130	7	3	1	93
'Seaton Park'	1967	S	110	5	7	5	110
'Trikkala'	1975	Y	112	5.5	6	2	81
'Karridale'	1985	S	139	7.5	6	2	127
'Denmark'	1992	S	142	7.5	5	2	141
'Leura'	1992	S	147	8	5	2	135
'Goulburn'	1992	S	141	7	5	5	196
'Gosse'	1992	Y	126	7	5	3	91
'Antas'	1999	B	138	7.8	1	3	100
'Campeda'	1999	S	123	6	6	5	123
'Napier'	2001	Y	140	7.5	6	5	88
'Coolamon'	2003	S	133	6.5	7	5	130
'Narrikup'	2009	S	126	6.5	7	3	185
'Rosabrook'	2009	S	142	7.5	6	5	161
'Monti'	2013	Y	110	5.5	6	2	101

Notes: Seeds sown/m2 is a bare seed equivalent rate. Year = Year seed first sold/ date registered as an Australian cultivar. Subspecies: B, brachycalycinum; S, subterraneum; Y, yanninicum. Min. growing season length (months) is the minimum target environment for reliable seed set. Burr burial: 1, little or no burial; 9, strong burial. Relative hardseededness: 1, least hard; 10, most hard.

Table A.2 Selected Herbicides, Commercial Availability, and Alternative Products and Suppliers. Information compiled from the Novachem New Zealand Online Agrichemical Database (Novachem 2016)

Herbicide	Distributor	Pack Sizes	Active Ingredient	Alternative Product		
				Name	Pack Sizes	Distributor
2,4-DB	Dow AgroSciences (NZ) Limited	20 Litres	**400 g/L 2,4-DB**		N/A	
Basagran	BASF New Zealand Limited	10 litres	**480 g/L Bentazone**	AGPRO Bentazone	1, 5, 10 and 20 litres	AGPRO NZ Ltd
				Bentazone 480	1, 5, 20 and 200 litres	Grosafe Chemicals
				Broadstar	10 litres	Adama NZ Ltd
				Delete	10 litres	AgriSource
				Dictate 480	5 and 20 litres	Nufarm Limited
				Pasture Guard Bentazone	20 litres	Ravensdown
				Troy 480SL	10 litres	Adria Crop Protection
Glyphosate 360 (WeedMaster G360)	Nufarm Limited	1, 5, 20 and 200 litres	**360 g/L Glyphosate**	Agmax Glyphosate	20, 100 and 200 litres	Agmax Industries
				AGPRO Green Glyphosate 360	5, 20, 100, 200 and 1000 litres	AGPRO NZ Ltd
				AGPRO Glyphosate 360	5, 20, 100 and 200 litres	AGPRO NZ Ltd
				Country Mile Glyphosate 360	1, 5, 20, 200 and 1000 litres	RD 1
				Hortcare Glyphosate 360 SC		GroSafe Chemicals
				Ken-Up Aquatic 360	20, 200 and 1000 litres	Kenso NZ Ltd
				Klin-Up Weedkiller	20 litres	Primehort Distributors
				Lion Herbicide	20 litres	PGG Wrightson Ltd.
				Orion Glyphosate 360	1, 5, 20, 200 and 1000 litres	Orion AgriScience
				Polaris 360	20 litres	Adama NZ Ltd
				Pro-Active Glyphosate 360	20, 200 and 1000 litres	Pro-Active
				Rainbow & Brown Glyphosate		Rainbow & Brown
				Ravensdown Glyphosate G360	20 litres	Ravensdown Agrochemicals
				Roundup 360 Pro	20, 100 and 1000 litres	Sinochem
				Roundup Renew	20, 100 and 1000 litres	Sinochem
				Synergy Glyphosate 360	20, 200 and 1000 litres	Orion AgriScience
Headstart	Zelam Ltd	5 and 20 litres	**50 g/litre flumetsulam**		N/A	
Jaguar	Bayer CropScience	10 Litres	**250 g/L Bromoxynil + 25 g/L Diflufenican**	Minder		Nufarm Limited
MCPB	Dow AgroSciences (NZ) Limited	5 and 20 litres	**385 g/L MCPB**	Helion	1, 5, 20 and 200 litres	Kenso NZ Ltd
				Nufarm MCPB	20 and 200 litres	Nufarm Limited
				Soft Touch	20 litres	Orion AgriScience
Pulsar	BASF New Zealand Limited	10 litres	**200 g/L MCPB + 200 g/L Bentazone**		N/A	
Spinnaker	BASF New Zealand Limited	1 litre	**240 g/L Imazethapyr**		N/A	
Sharpen	BASF New Zealand Limited	150, 500 g and 1 kg	**700 g/kg Saflufenacil**		N/A	

Table A.3 Chemical structures for active ingredients of herbicides used in all experiments

		
Chemical structure of 2,4-DB, from Kirby (1980)	Chemical structure of MCPB, from (PubChem 2016d).	Chemical structure of bentazone (Bentazone), from PubChem (2016b).
		
Chemical structure of glyphosate, from (PubChem 2016c)	Chemical structure of imazethapyr (Imazethapyr), from PubChem (2016e).	Chemical structure of flumetsulam, from PubChem (2016g)
		
Chemical structure of saflufenacil (Saflufenacil) from PubChem (2016h).	Chemical structure of bromoxynil (PubChem, 2004).	Chemical structure of diflufenican, from (PubChem 2016f)

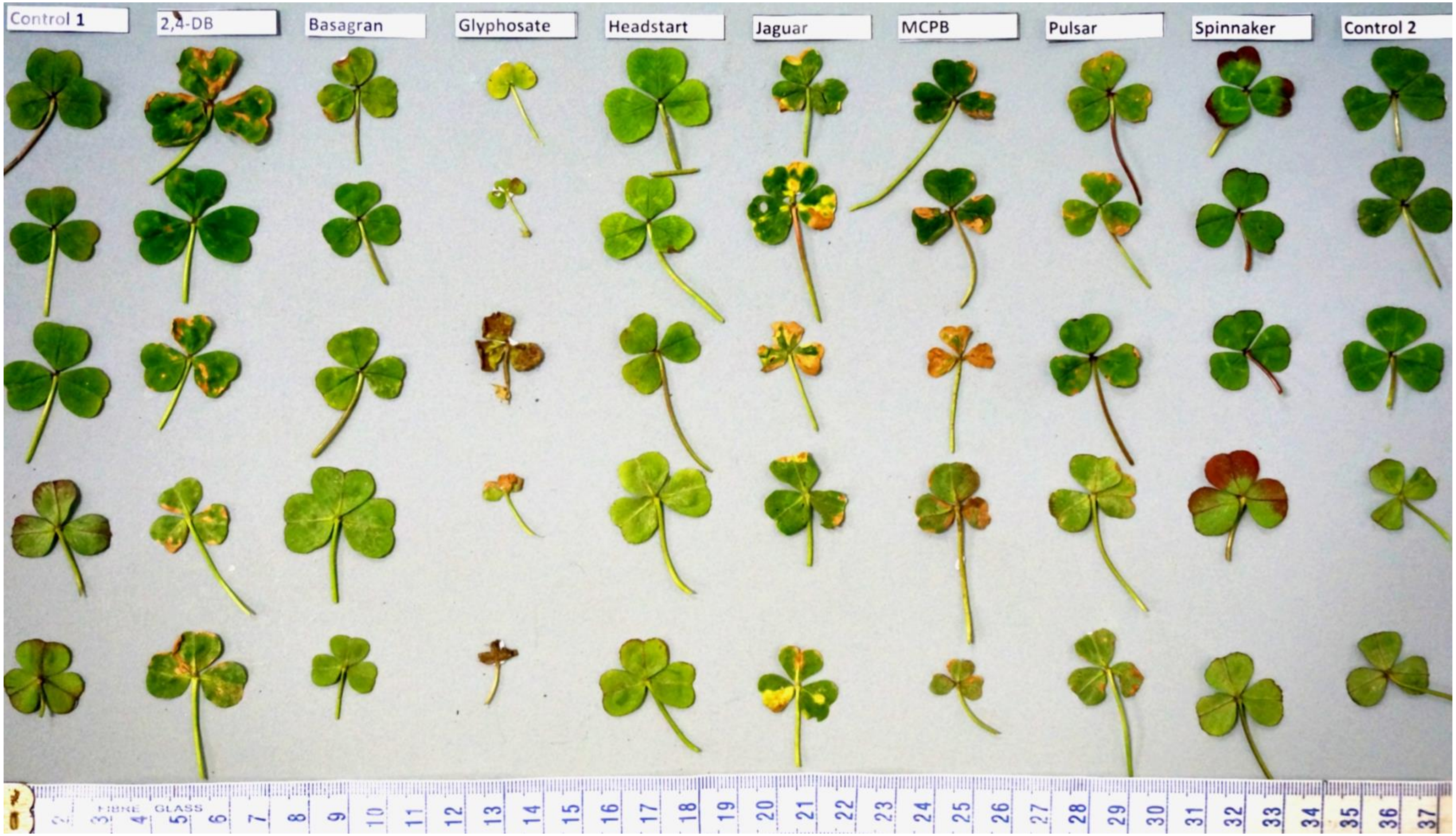


Plate A.1 Difference in leaf phytotoxicity symptoms of subterranean clover cultivar 'Antas' to all herbicide treatments 30 DAA, on the 14 July 2016 at Ashley Dene, Springston, Canterbury, New Zealand.

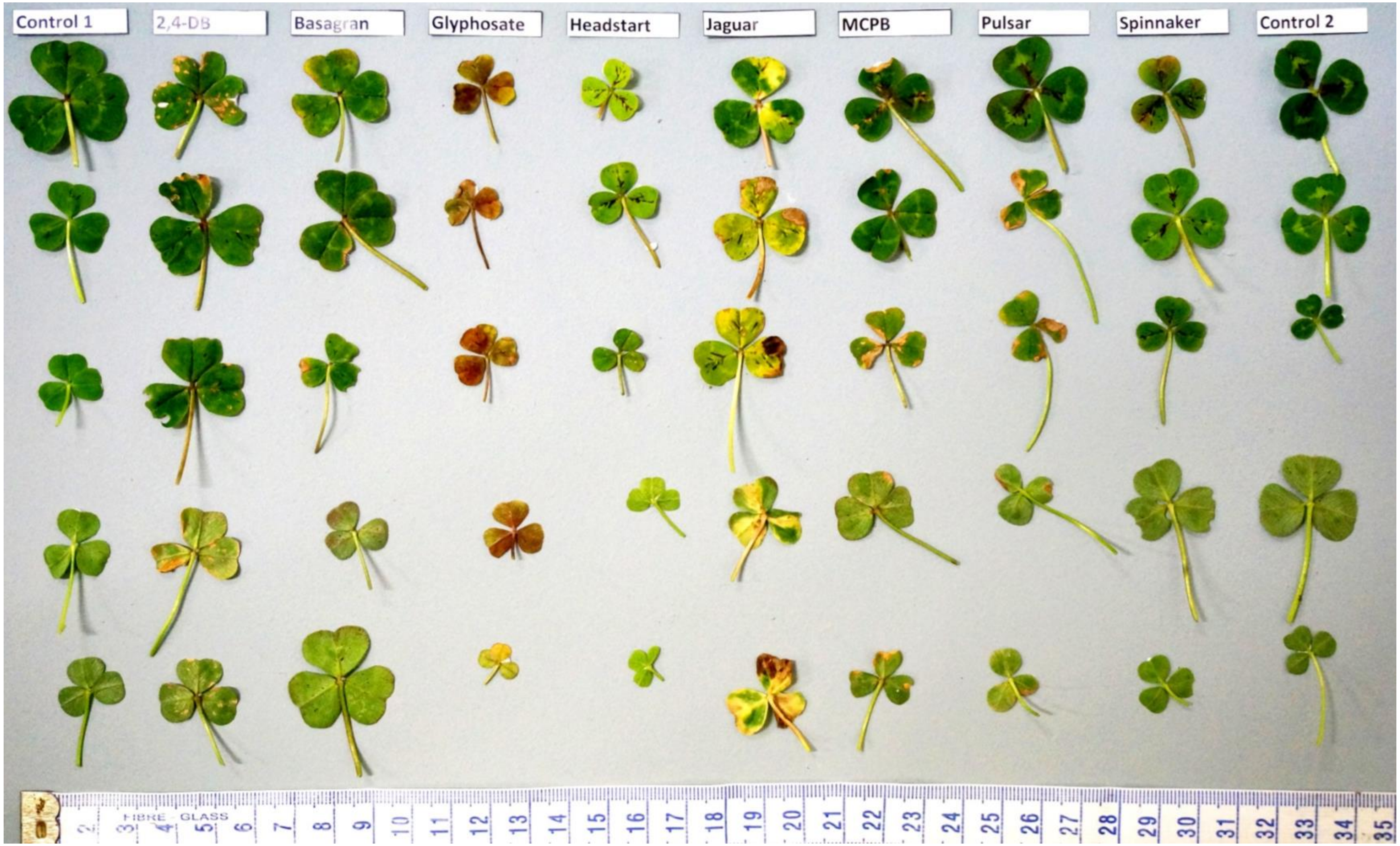


Plate A.2 Difference in leaf phytotoxicity symptoms of subterranean clover cultivar 'Denmark' to all herbicide treatments 30 DAA, on the 14 July 2016 at Ashley Dene, Springston, Canterbury, New Zealand.

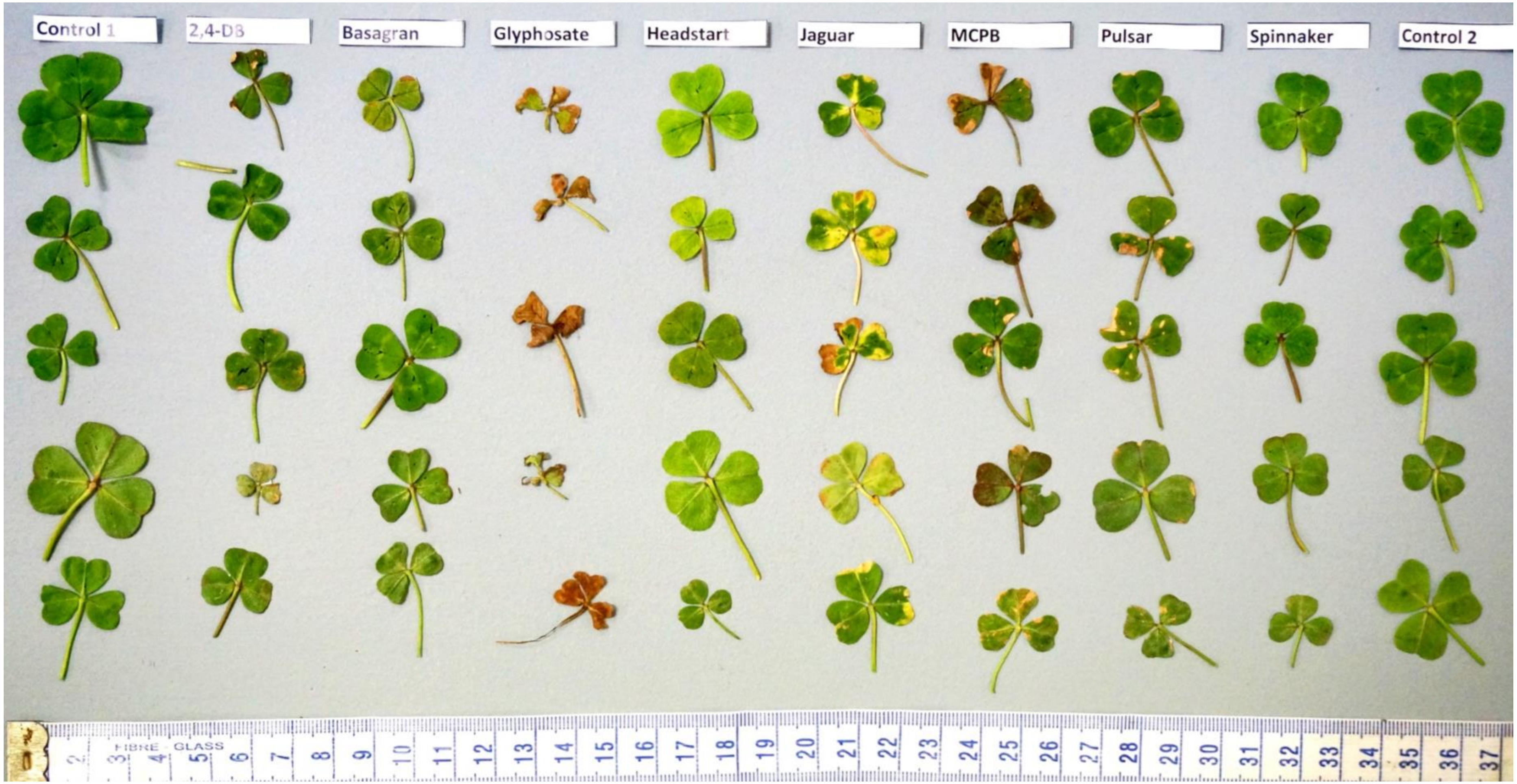


Plate A.3 Difference in leaf phytotoxicity symptoms of subterranean clover cultivar 'Monti' to all herbicide treatments 30 DAA, on the 14 July 2016 at Ashley Dene, Springston, Canterbury, New Zealand.